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PATENT & TRADEMARK OFFICE

FIG. 1.

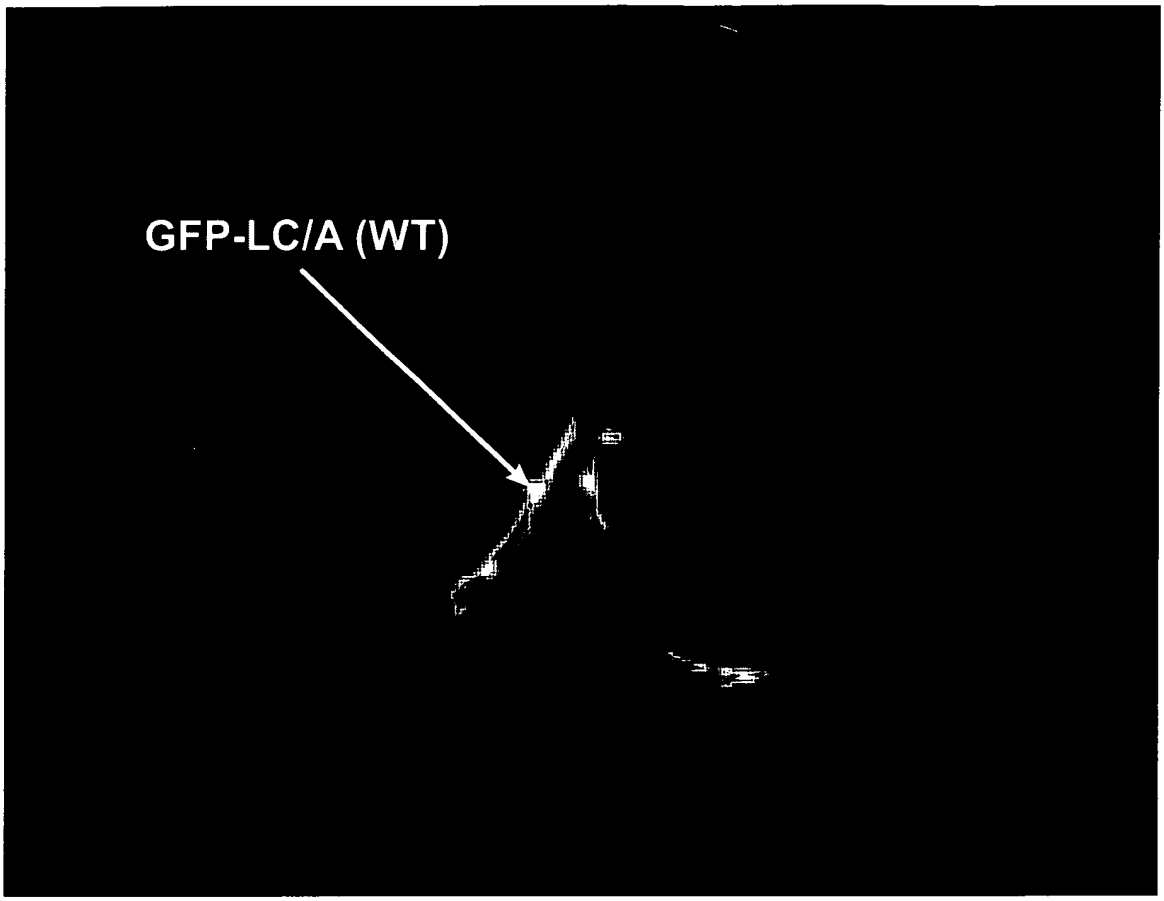

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FIG. 2.

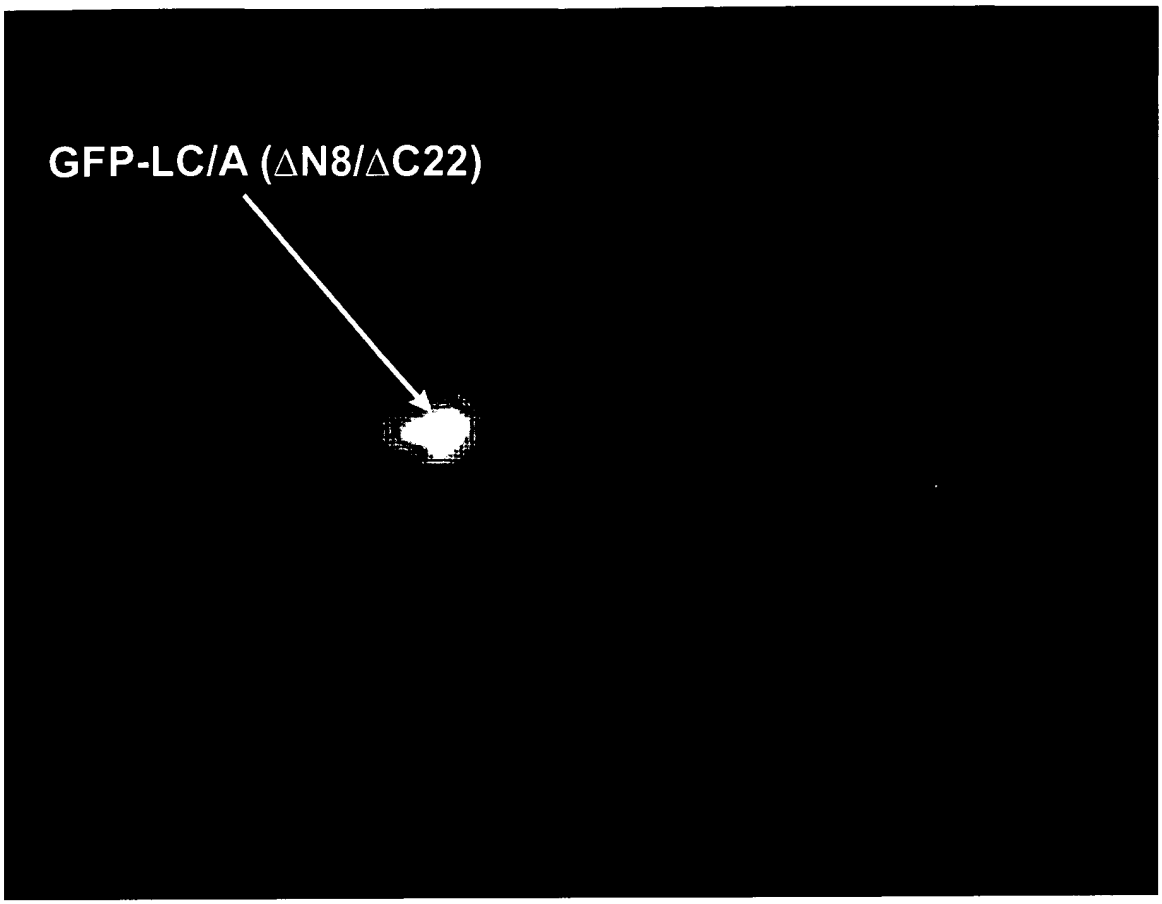


FIG. 3.

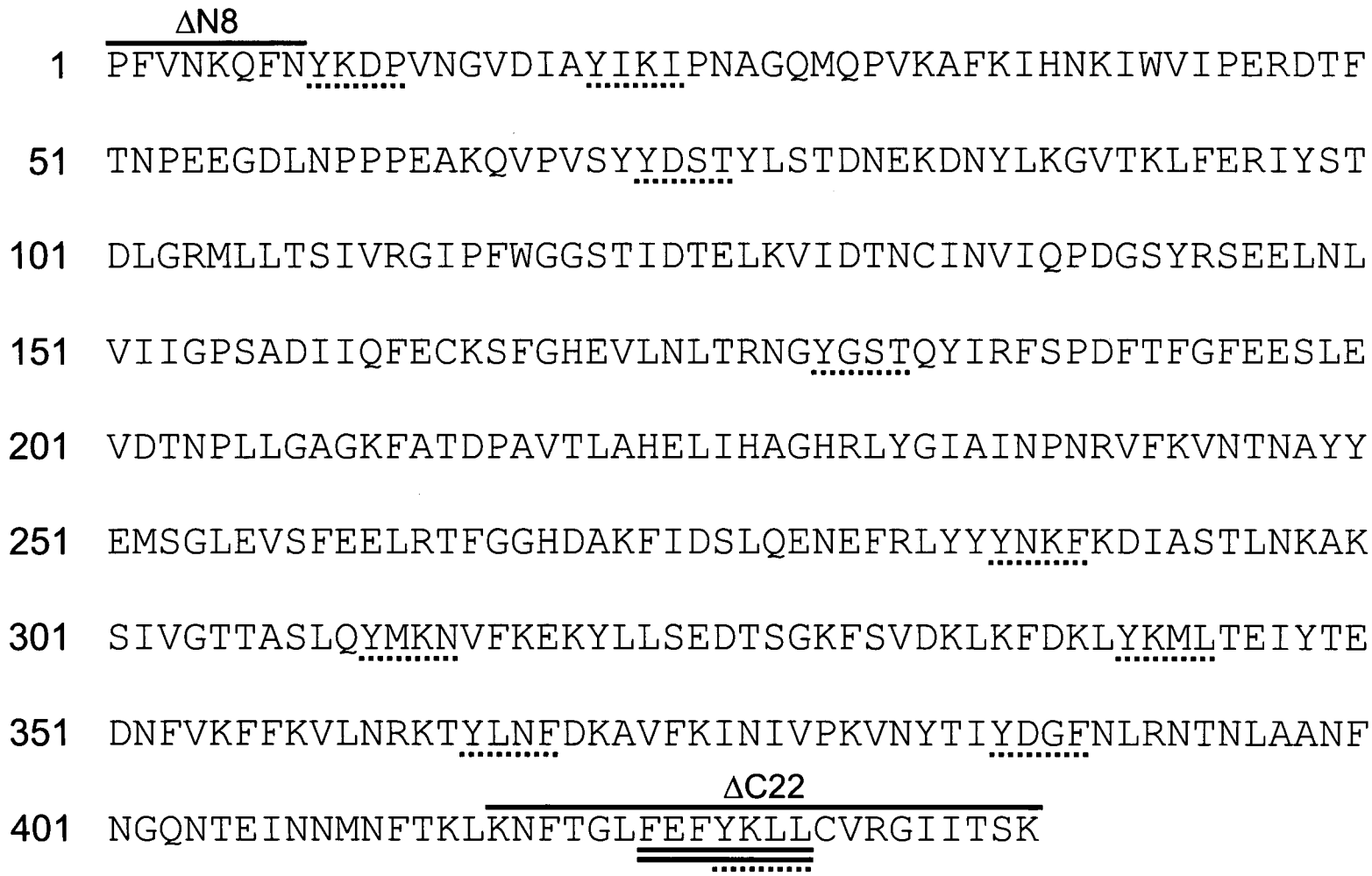

ΔN8
1 PFVNKQFNYKDPVNGVDIAYIKIPNAGQMQPVKAFKIHNKIWVIPERDTF
51 TNPEEGDLNPPPEAKQVPVSYYDSTYLSTDNEKDNYLKGVTKLFERIYST
101 DLGRMLLTSIVRGIPFWGGSTIDTELKVIDTNCINVIQPDGSYRSEELNL
151 VIIGPSADIIQFECKSFGHEVLNLTRNGYGSTQYIRFSPDFTFGFEESLE
201 VDTNPLLGAGKFATDPAVTLAHELIHAGHRLYGIAINPNRVFKVNTNAYY
251 EMSGLEVSFEELRTFGGHDAKFIDSLQENEFRLYYYNKFKDIASTLNKAK
301 SIVGTTASLQYMKNVFKEKYLLSEDTSGKFSVDKLKFDKLYKMLTEIYTE
351 DNFVKFFKVLNRKTYLNFDKAVFKINIVPKVNYTIYDGFNLRNTNLAANF
ΔC22
401 NGQNTEINNMNFTKLKNFTGLFEFYKLLCVRGIITSK

FIG. 4.

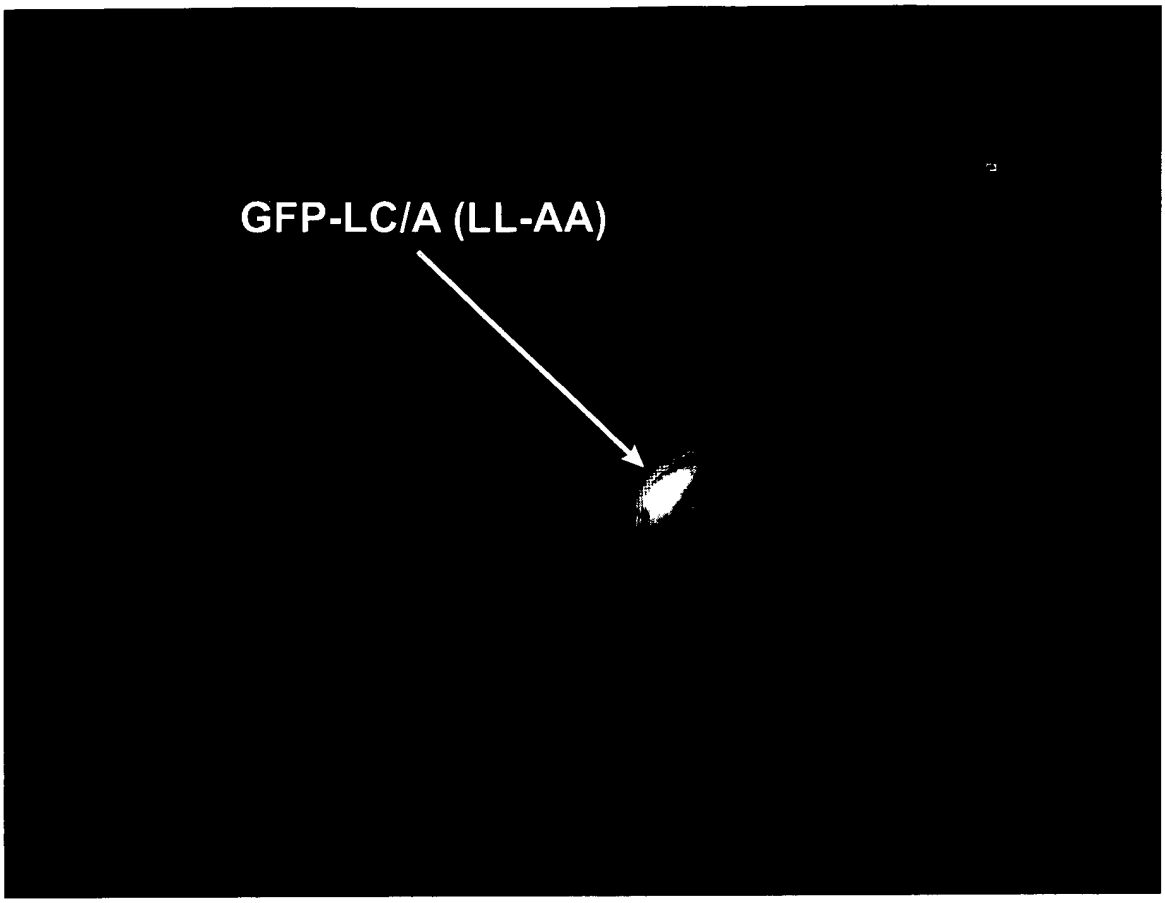


FIG. 5.

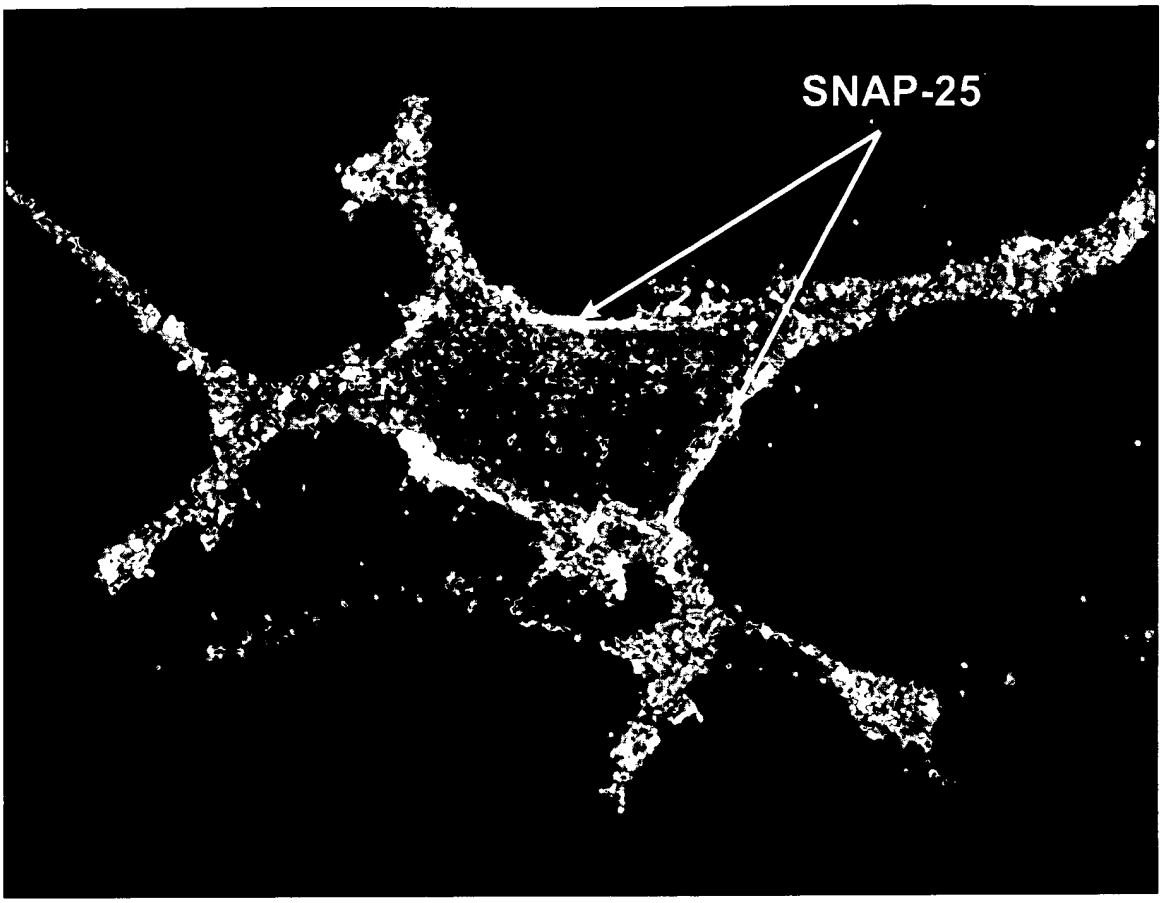


FIG. 6.

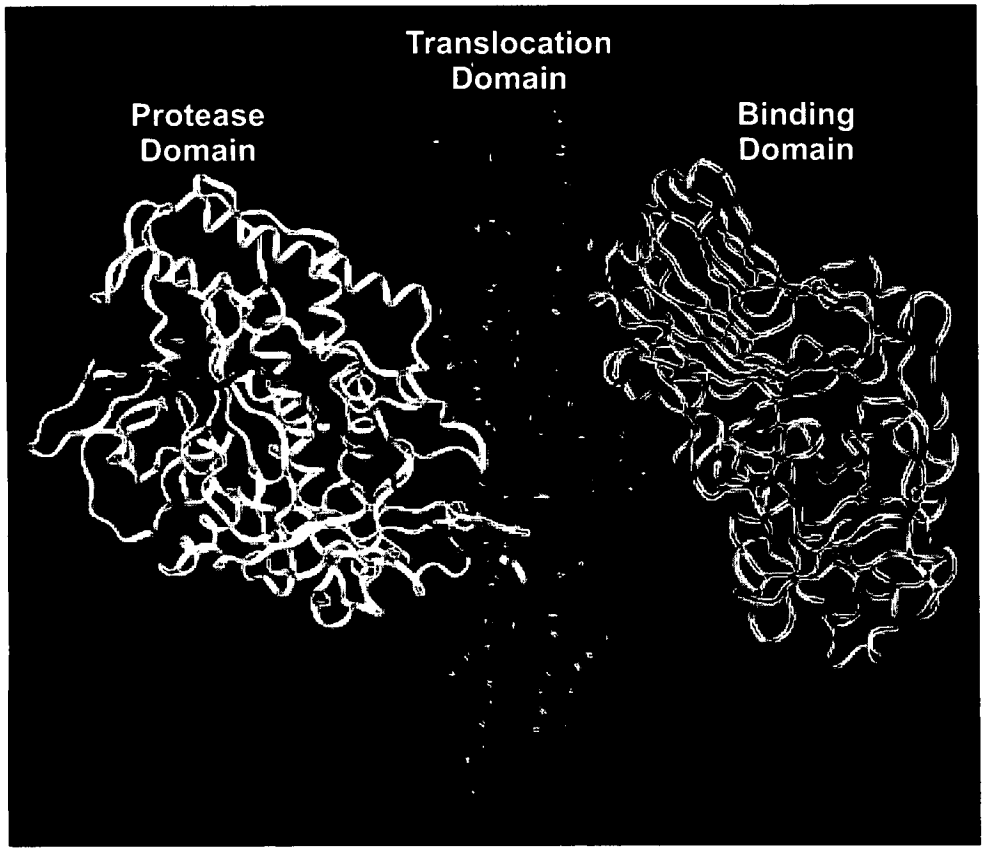


FIG. 7.

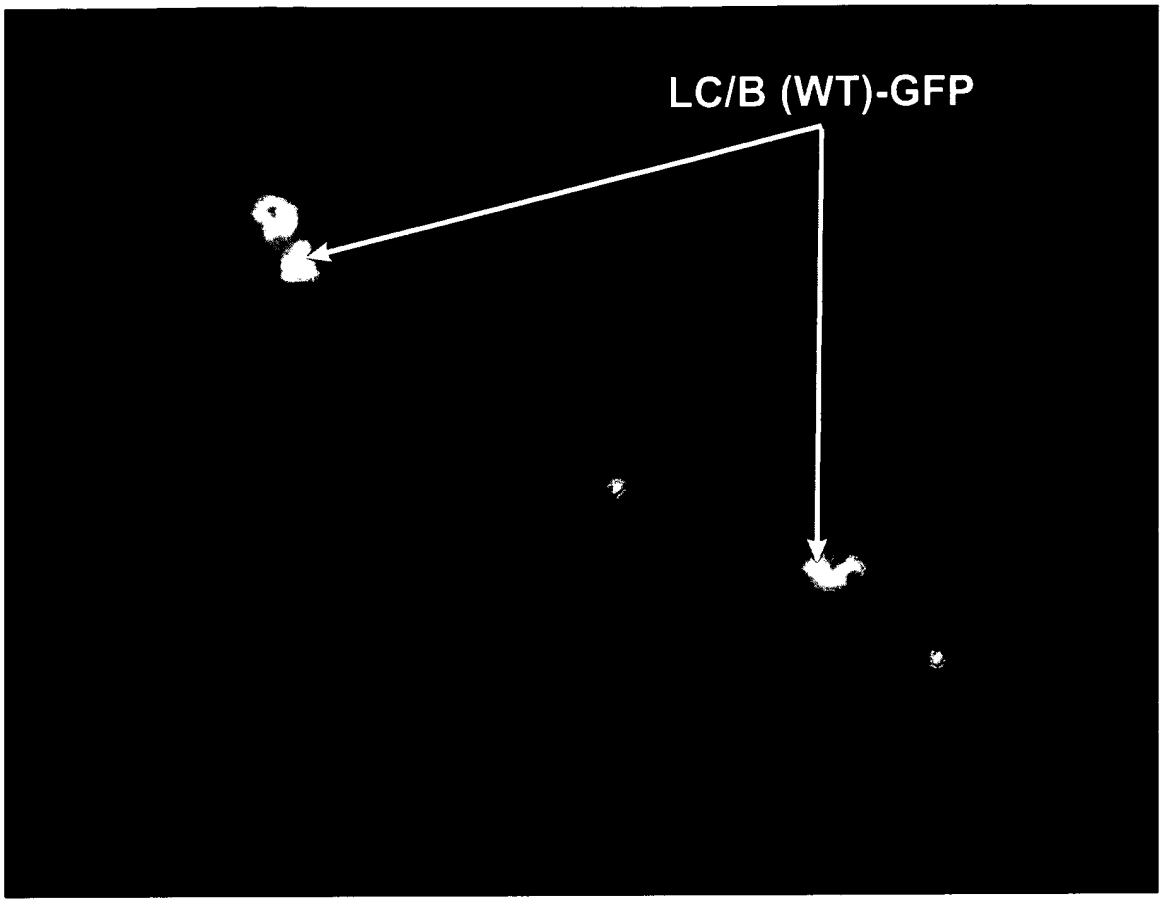


FIG. 8.

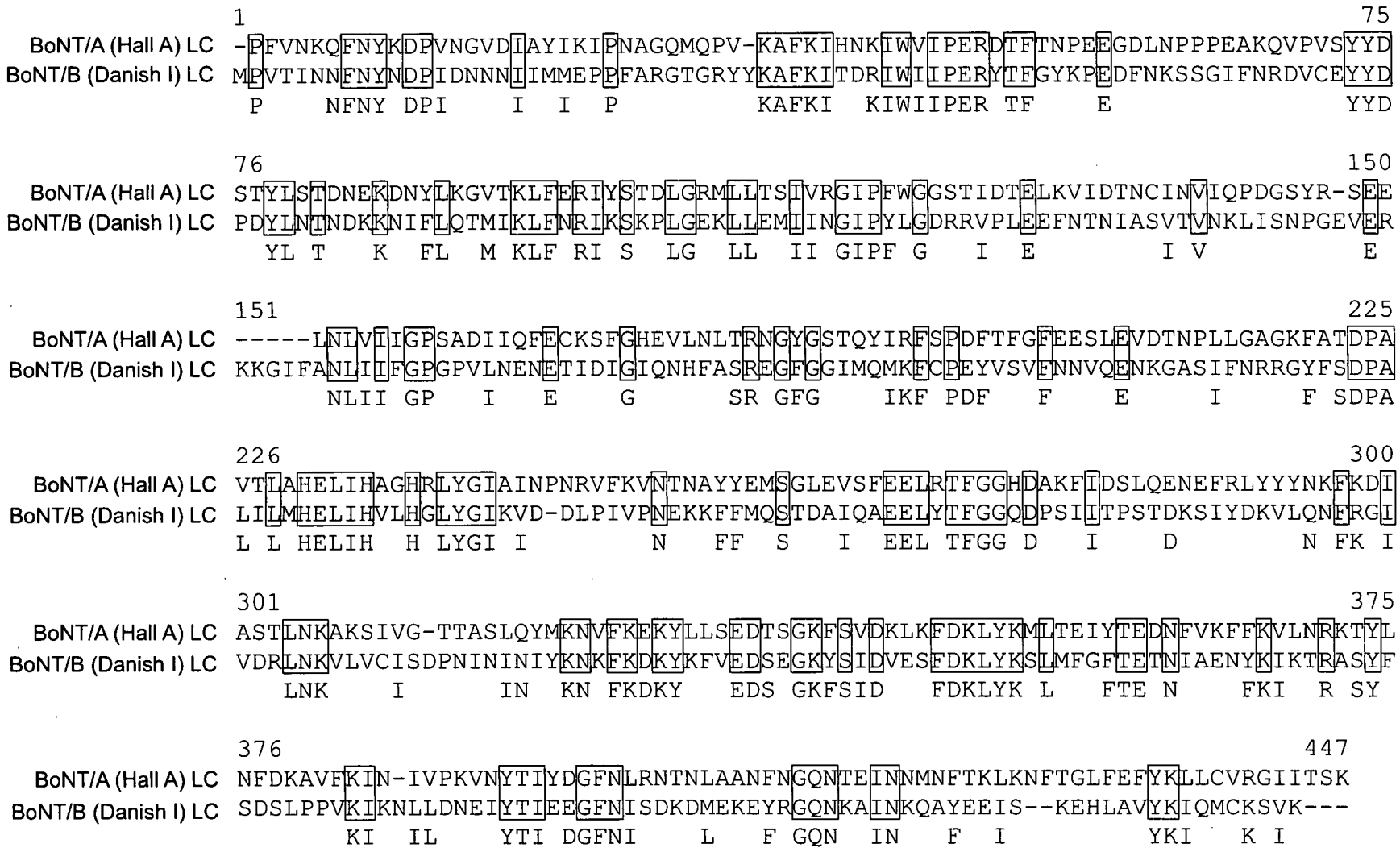

1 75
BoNT/A (Hall A) LC -PFVNKQFNYKDPVNGVDIAYIKIPNAGQMQPV-KAFKIHNKIWVIPERDTFTNPEEGDLNPPPEAKQVPVSYYD
BoNT/B (Danish I) LC MPVTINNFNYNDPIDNNNIIMMEPPFARGTGRYYKAFKITDRIWIIPERYTFGYKPEDFNKSSGIFNRDVCEYYD
P NFNY DPI I I P KAFKI KIWIIPER TF E YYD
76 150
BoNT/A (Hall A) LC STYLSTDNEKDNYLKGVTKLFERIYSTDLGRMLLTSIVRGIPFWGGSTIDTELKVIDTNCINVIQPDGSYR-SEE
BoNT/B (Danish I) LC PDYLNTINDKKNIFLQTMIKLFNRIKSKPLGEKLLEMIINGIPYLGDRRVPLEEFNTNIASVTVNKLISNPGEVER
YL T K FL M KLF RI S LG LL II GIPF G I E I V E
151 225
BoNT/A (Hall A) LC -----LNLVIIGPSADIIQFECKSFGHEVLNLTRNGYGSTQYIRFSPDFTFGFEESLEVDTNPLLGAGKFATDPA
BoNT/B (Danish I) LC KKGIFANLIIFGPGPVLNENETIDIGIQNHFASREGFGGIMQMKFCPEYVSVFNNVQENKGASIFNRRGYFSDPA
NLII GP I E G SR GFG IKF PDF F E I F SDPA
226 300
BoNT/A (Hall A) LC VTLAHELIHAGHRLYGIAINPNRVFKVNTNAYYEMSGLEVSFEELRTFGGHDAKFIDSLQENEFRLYYYNKFKDI
BoNT/B (Danish I) LC LILMHELIHVLHGLYGIKVD-DLPIVPNEKKFFMQSTDAIQAEELYTFGGQDPSIITPSTDKSIYDKVLQNFRGI
L L HELIH H LYGI I N FF S I EEL TFGG D I D N FK I
301 375
BoNT/A (Hall A) LC ASTLNKAKSIVG-TTASLQYMKNVFKEKYLLSEDTSGKFSVDKLKFDKLYKMLTEIYTEDNFVKFFKVLNRKTYL
BoNT/B (Danish I) LC VDRLNKVLVCISDPNININIYKNKFKDKYKFVEDSEGKYSIDVESFDKLYKSLMFGFTETNIAENYKIKTRASYF
LNK I IN KN FKDKY EDS GKFSID FDKLYK L FTE N FKI R SY
376 447
BoNT/A (Hall A) LC NFDKAVFKIN-IVPKVNYTIYDGFNLRNTNLAANFNGQNTEINNMNFTKLKNFTGLFEFYKLLCVRGIITSK
BoNT/B (Danish I) LC SDSLPPVKIKNLLDNEIYTIEEGFNISDKDMEKEYRGQNKAINKQAYEEIS--KEHLAVYKIQMCKSVK---
KI IL YTI DGFNI L F GQN IN F I YKI K I

FIG. 9.

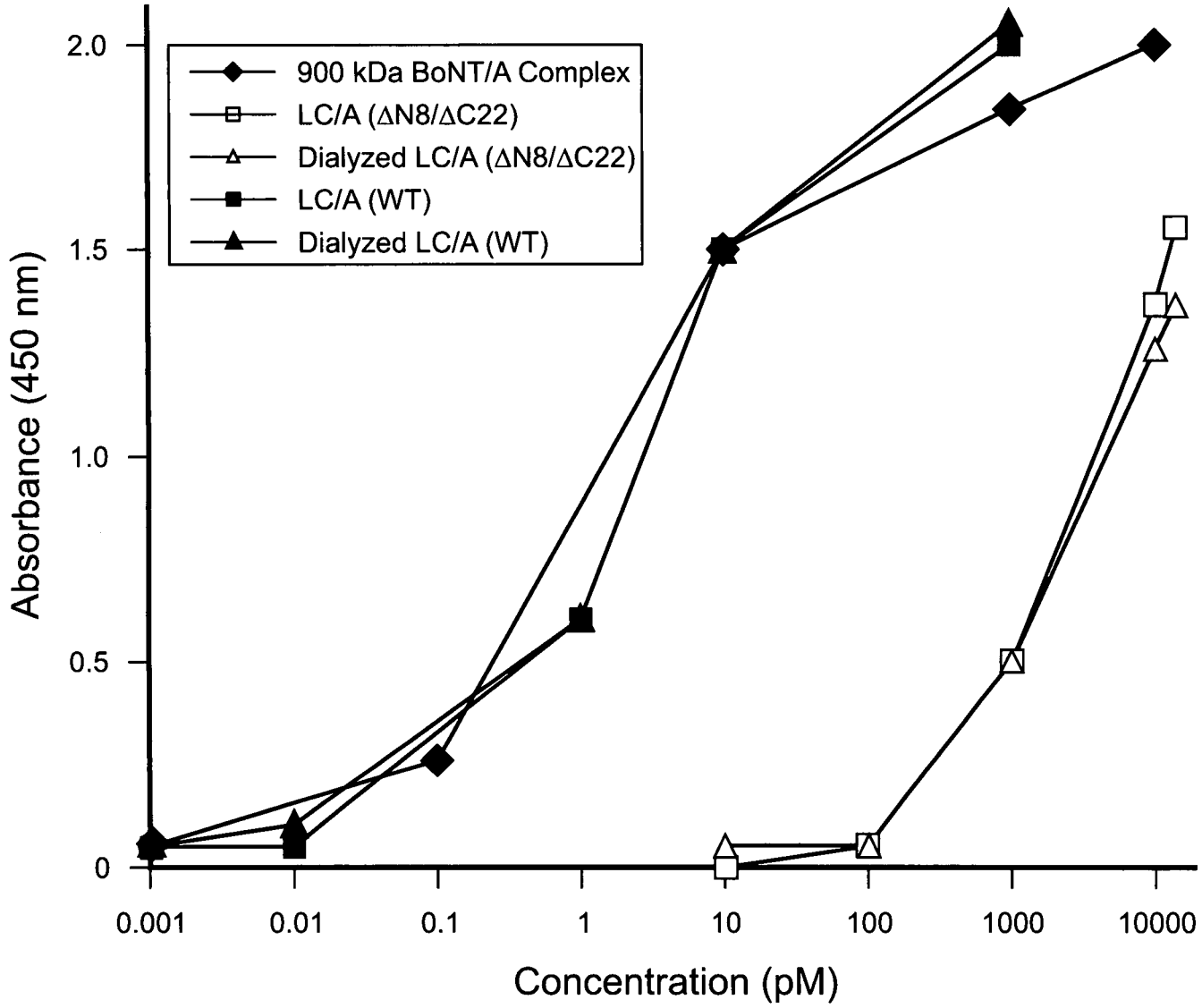

900 kDa BoNT/A Complex
LC/A (ΔN8/ΔC22)
Dialyzed LC/A (ΔN8/ΔC22)
LC/A (WT)
Dialyzed LC/A (WT)
Absorbance (450 nm)
2.0
1.5
1.0
0.5
0
0.001
0.01
0.1
1
10
100
1000
10000
Concentration (pM)

FIG. 10.

Amino Terminus
6 x His
S-tag
ΔN8
LC/A (WT)
PFVNKQFNYKDPV---
LC/A (ΔN8/ΔC22)
MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAM---------YKDPV---
N-His-LC/A (WT)
MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAMGSFVNKQFNYKDPV---
Carboxy Terminus
ΔC22
6 x His
LC/A (WT)
---NFTKLKNFTGLFEFYKLLCVRGIITSK
LC/A (ΔN8/ΔC22)
---NFTKL----------TRAHHHHHH
N-His-LC/A (WT)
---NFTKLKNFTGLFEFYKLLCVRGIITSK
Leucine-based
Motif

FIG. 11.

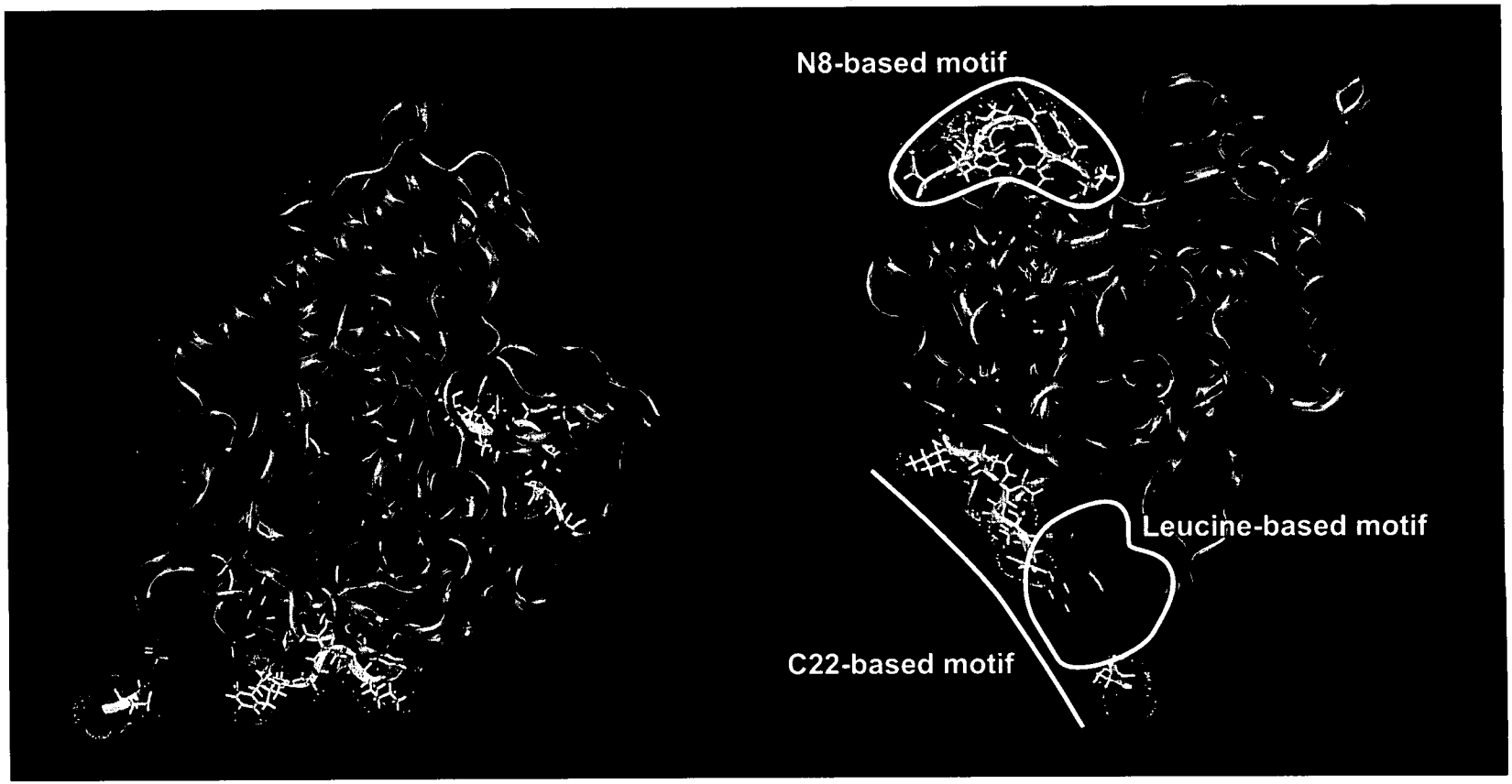


FIG. 12.

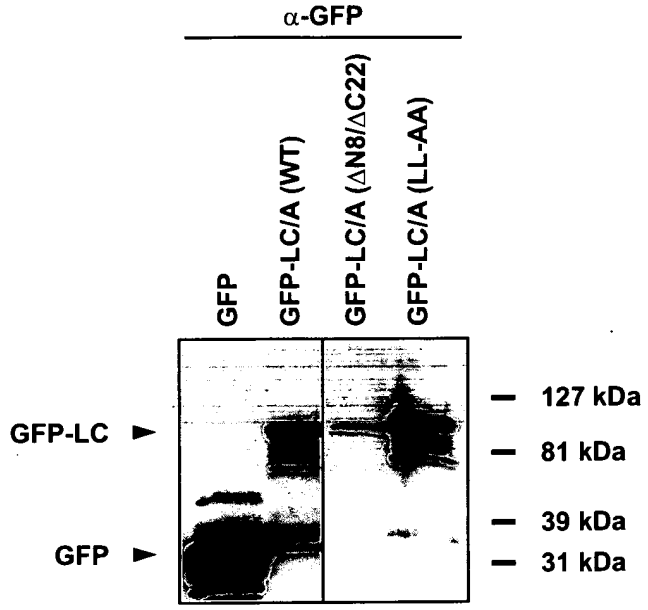

α-GFP
GFP
GFP-LC/A (WT)
GFP-LC/A (ΔN8/ΔC22)
GFP-LC/A (LL-AA)
GFP-LC
GFP
127 kDa
81 kDa
39 kDa
31 kDa

FIG. 13.

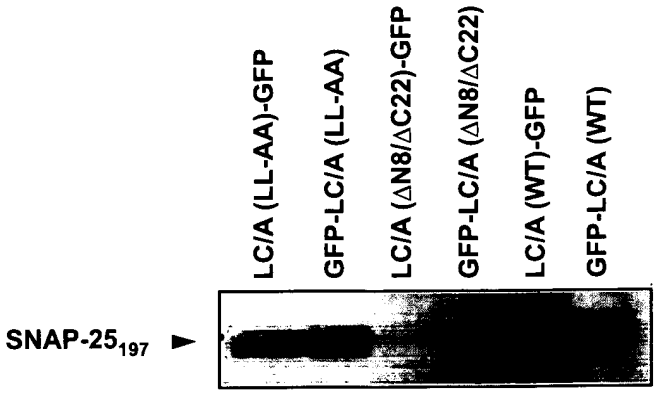

LC/A (LL-AA)-GFP
GFP-LC/A (LL-AA)
LC/A (ΔN8/ΔC22)-GFP
GFP-LC/A (ΔN8/ΔC22)
LC/A (WT)-GFP
GFP-LC/A (WT)
SNAP-25197

FIG. 14.

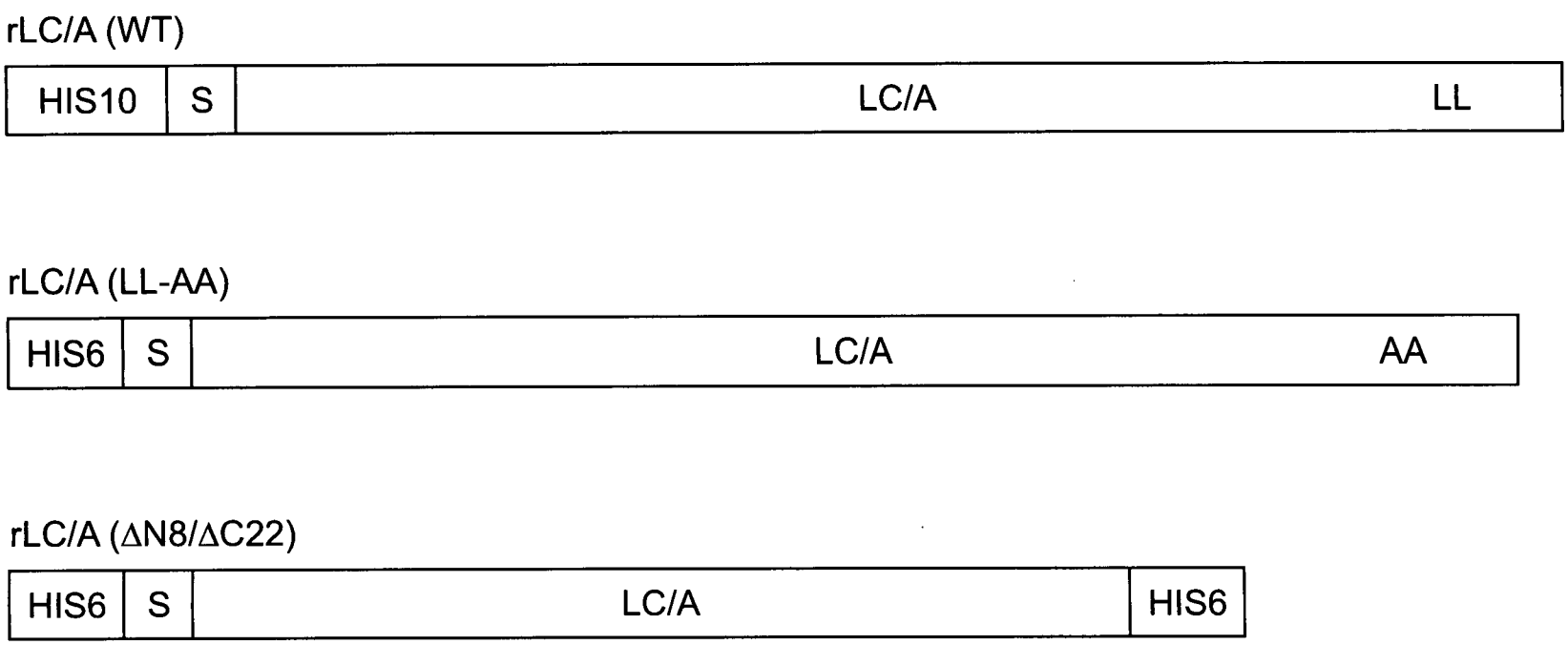

rLC/A (WT)
HIS10
S
LC/A
LL
rLC/A (LL-AA)
HIS6
S
LC/A
AA
rLC/A (ΔN8/ΔC22)
HIS6
S
LC/A
HIS6

FIG. 15.

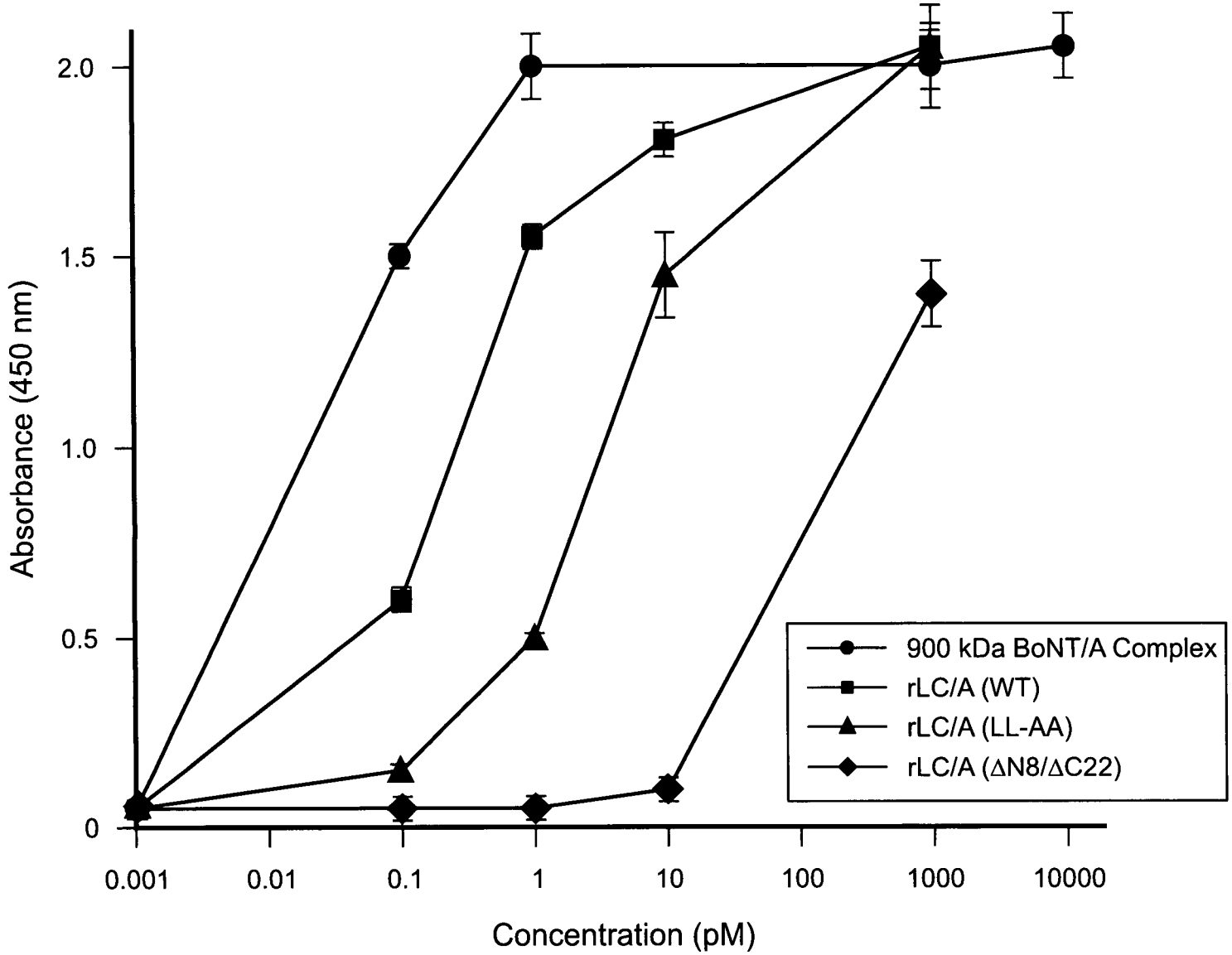

Absorbance (450 nm)
2.0
1.5
1.0
0.5
0
0.001
0.01
0.1
1
10
100
1000
10000
Concentration (pM)
900 kDa BoNT/A Complex
rLC/A (WT)
rLC/A (LL-AA)
rLC/A (ΔN8/ΔC22)

FIG. 16.

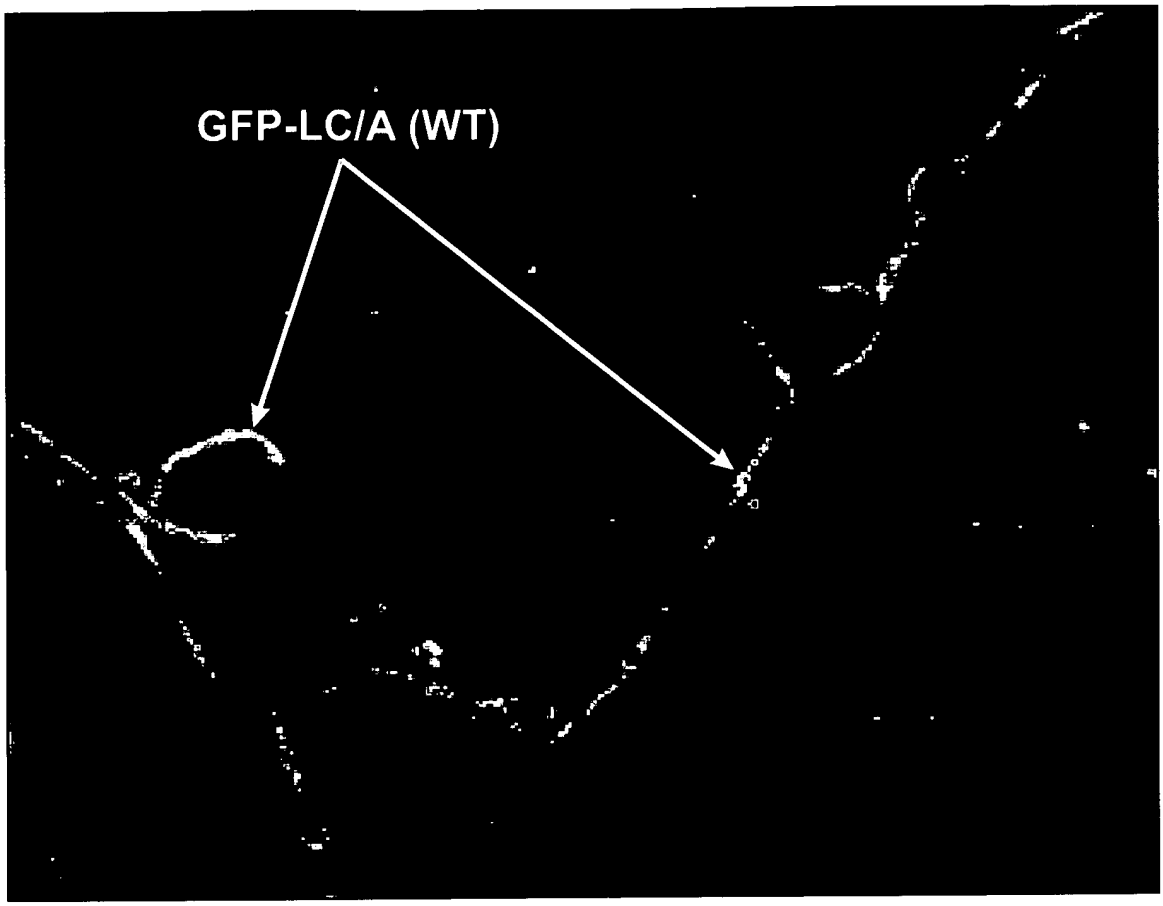


FIG. 17.

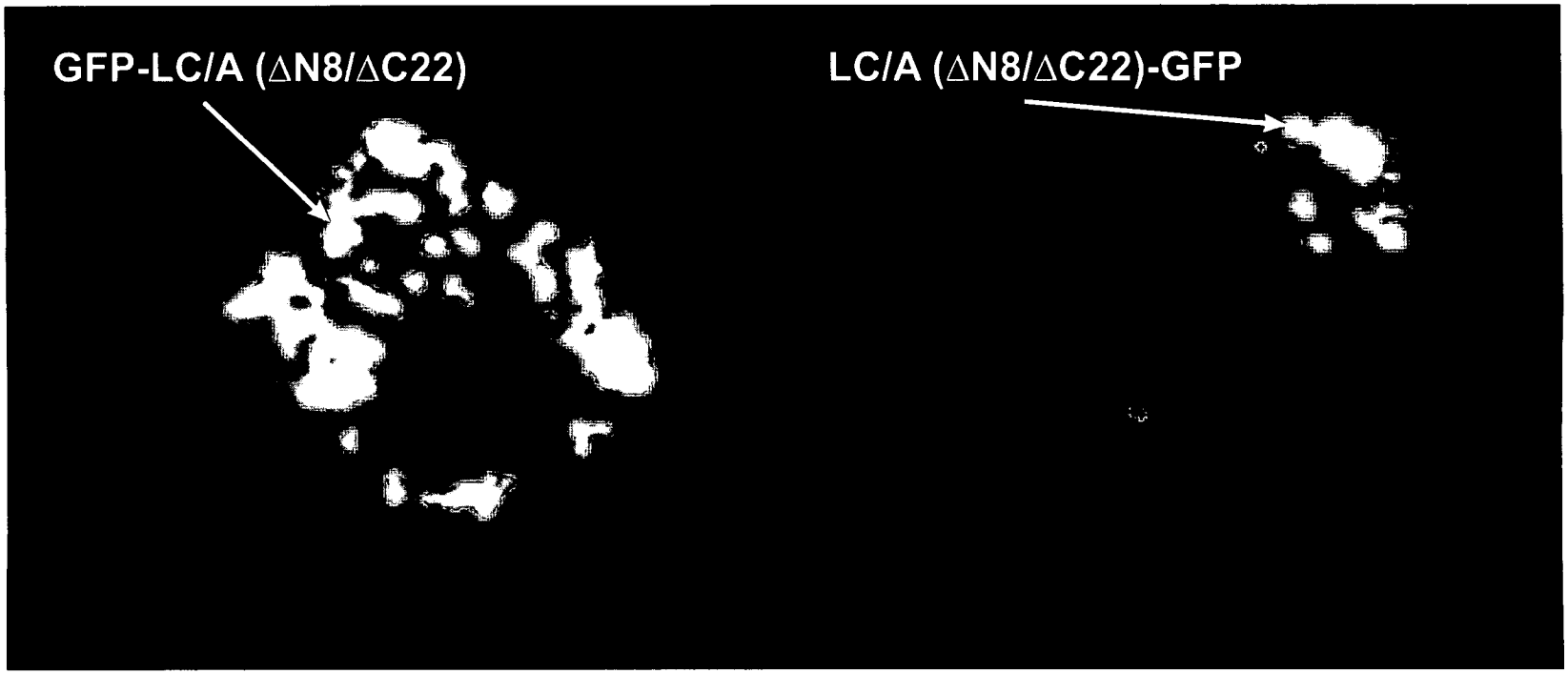


FIG. 18.

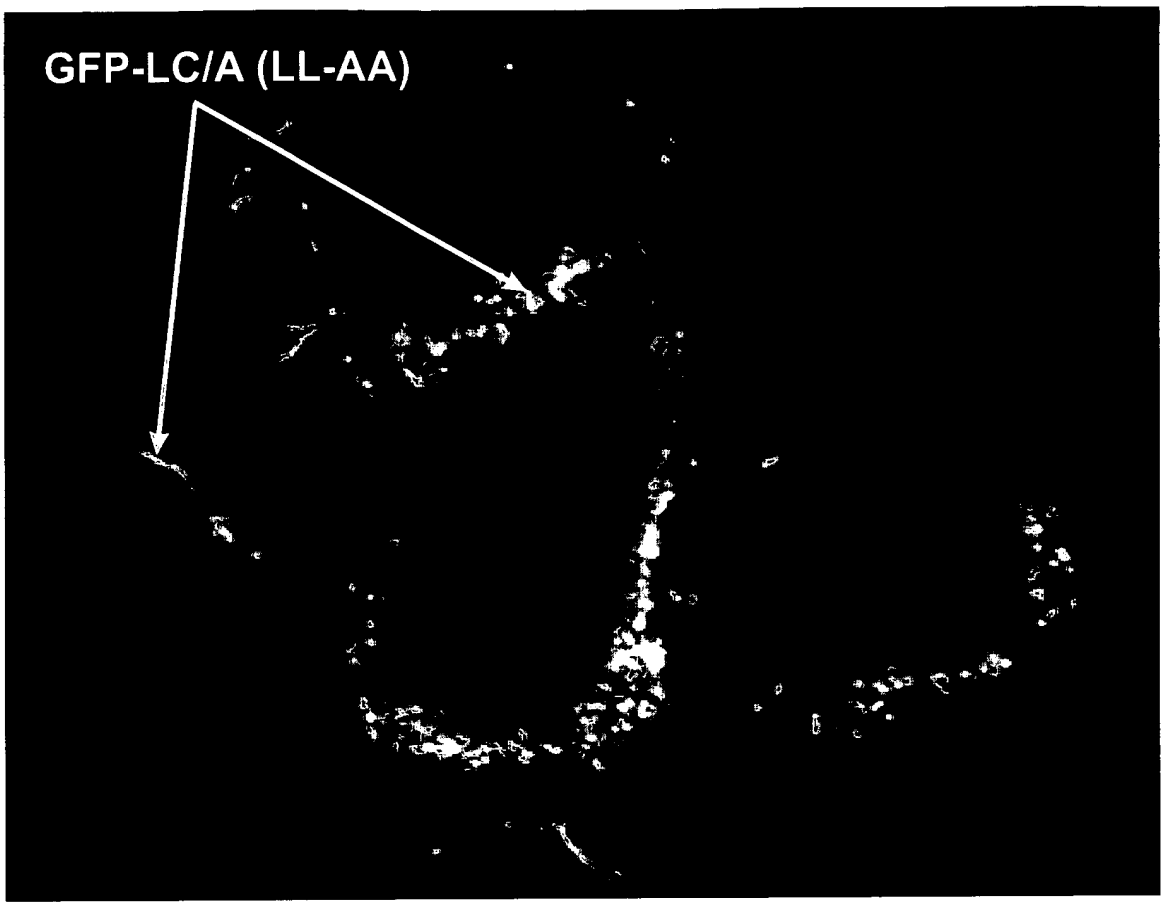


FIG. 19.

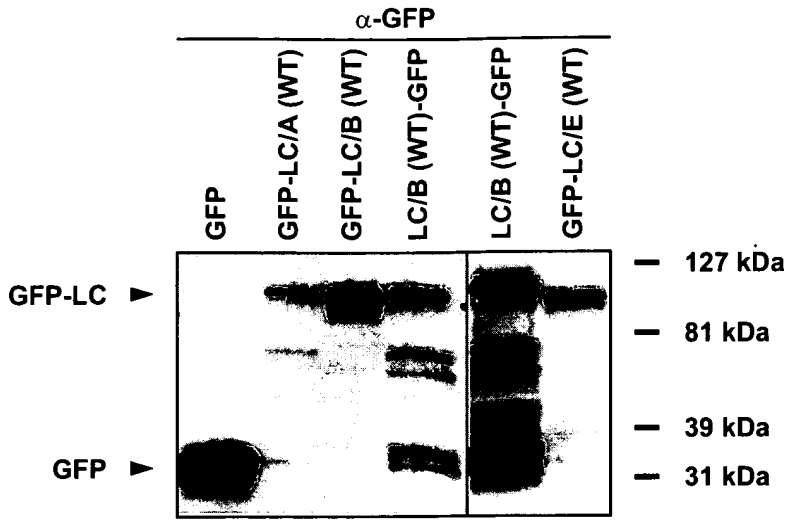

α-GFP
GFP
GFP-LC/A (WT)
GFP-LC/B (WT)
LC/B (WT)-GFP
LC/B (WT)-GFP
GFP-LC/E (WT)
GFP-LC
GFP
127 kDa
81 kDa
39 kDa
31 kDa

FIG. 20A.

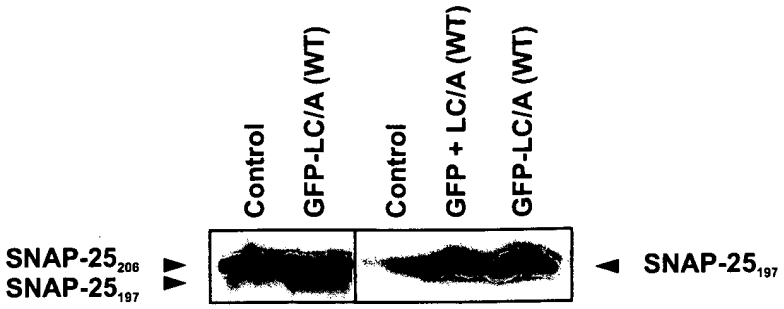


FIG. 20B.

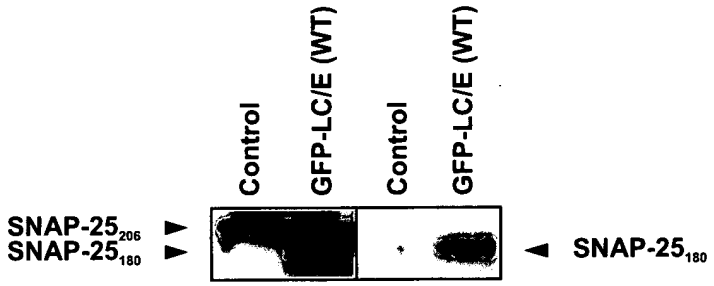


FIG. 21.

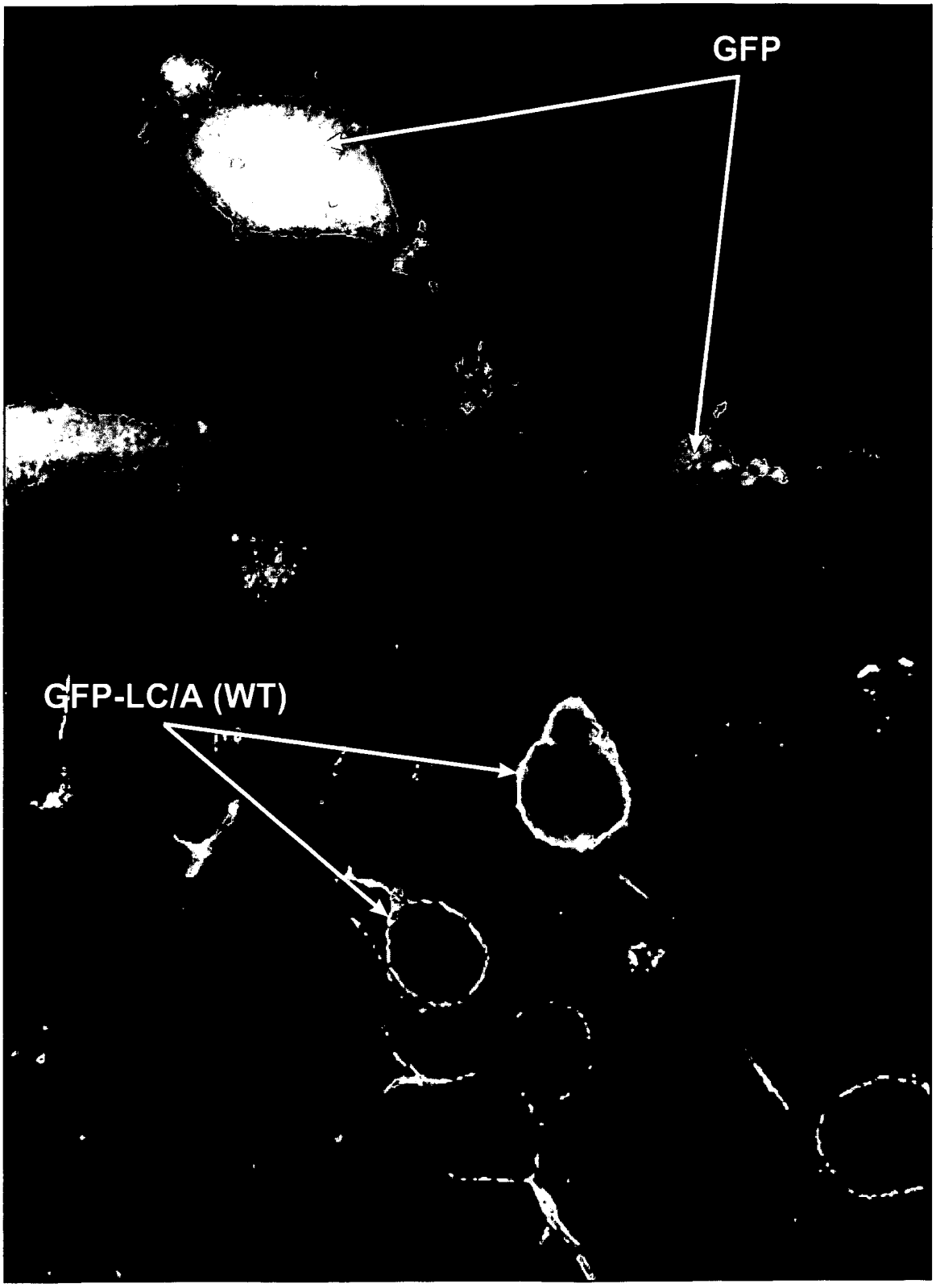


FIG. 22.

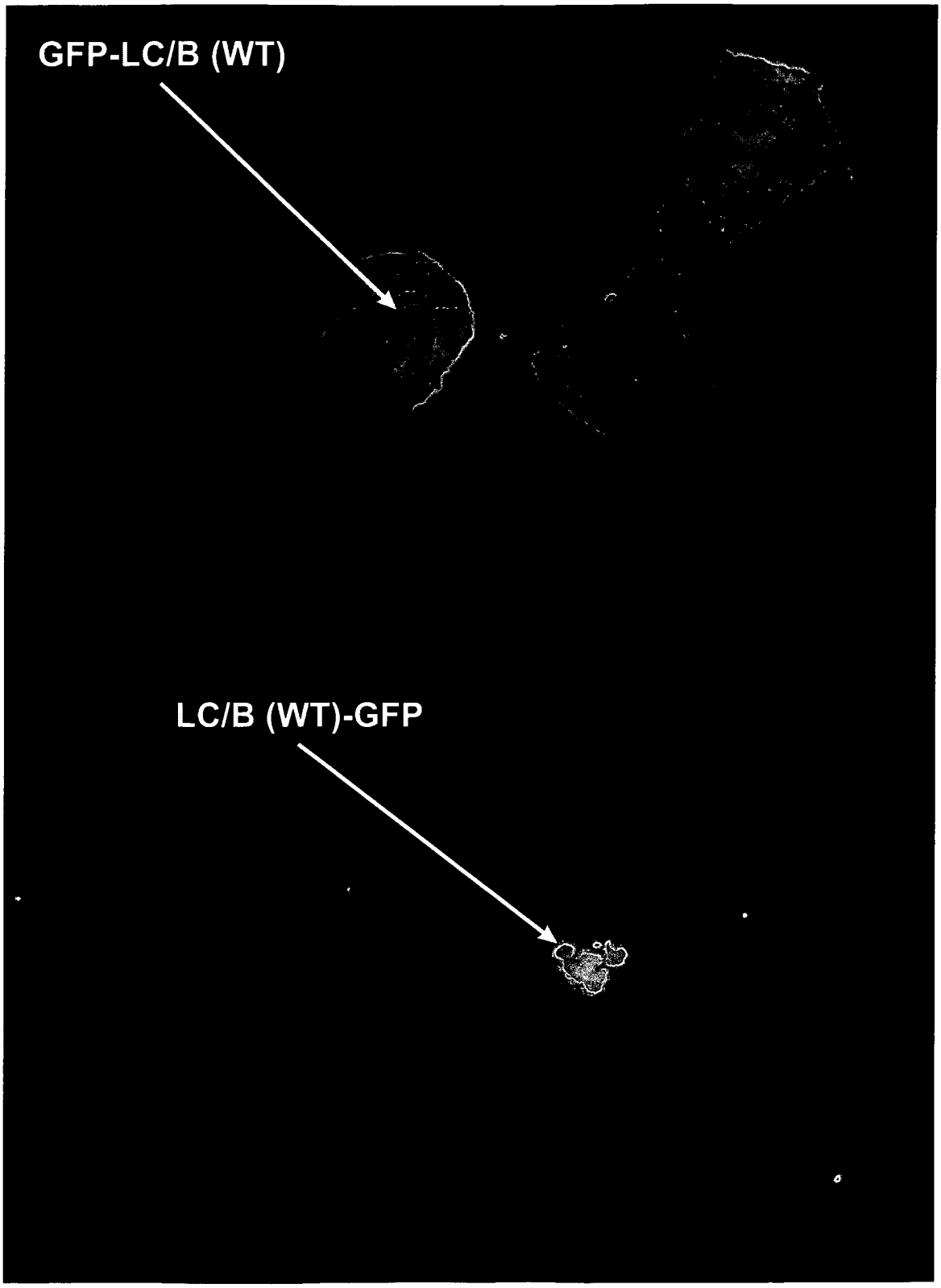


FIG. 23.

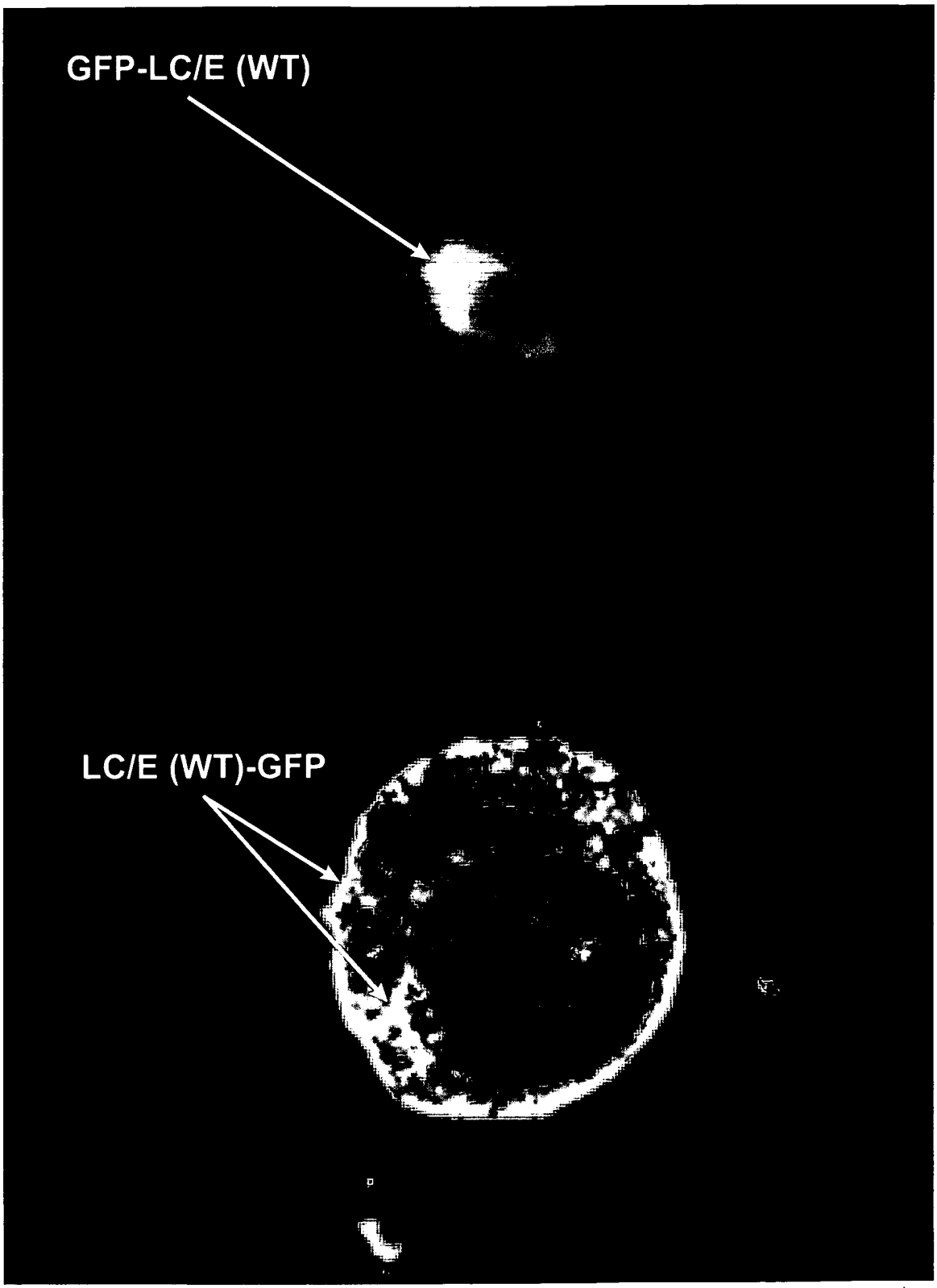


FIG. 24A.

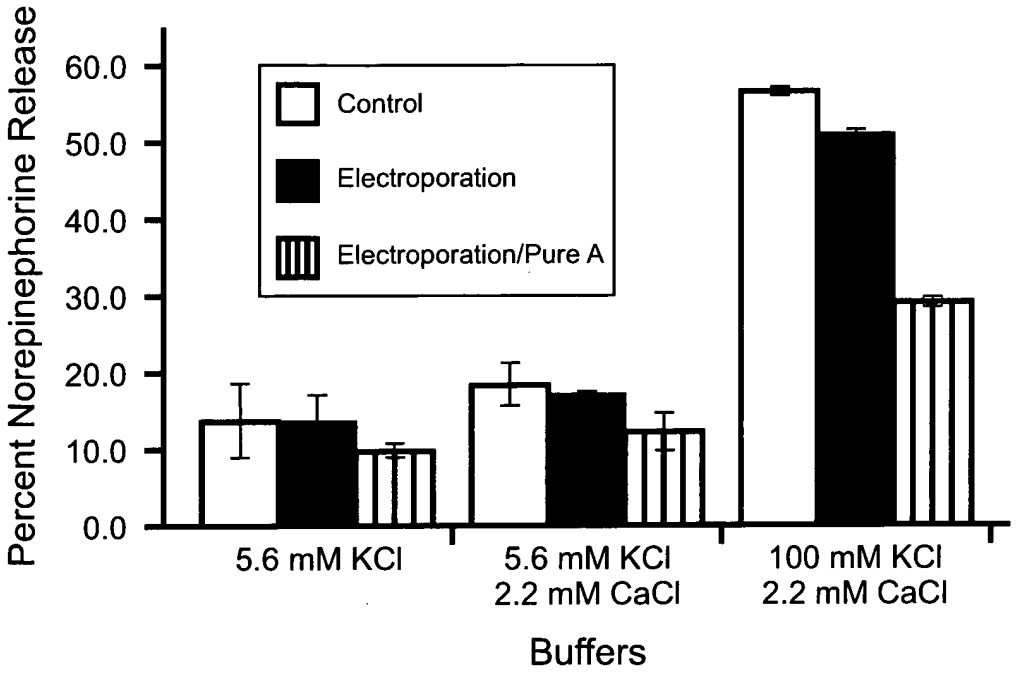


FIG. 24B.

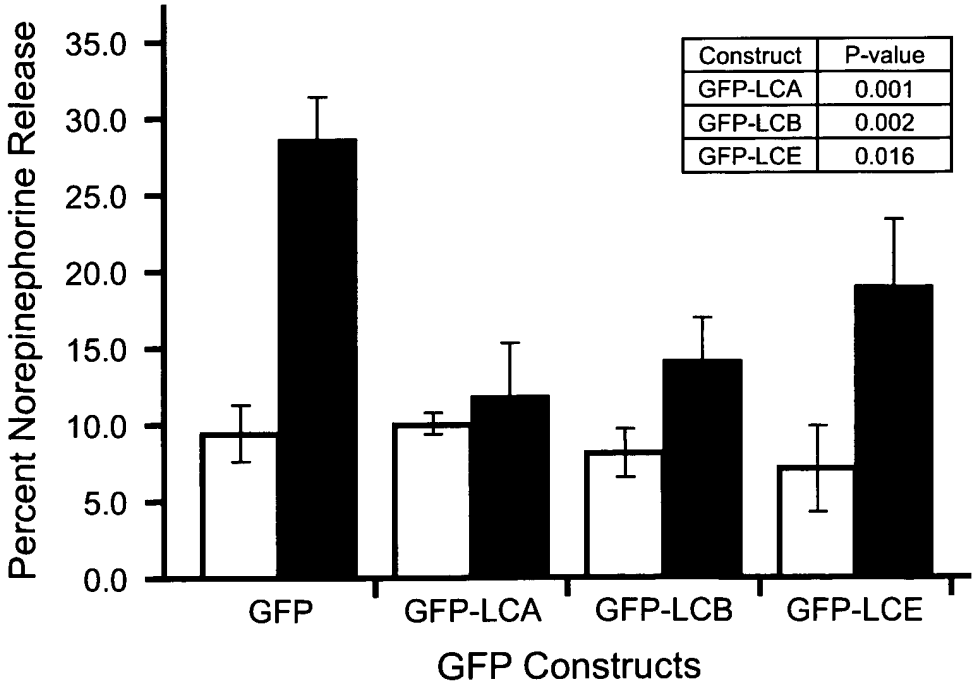


FIG. 25.

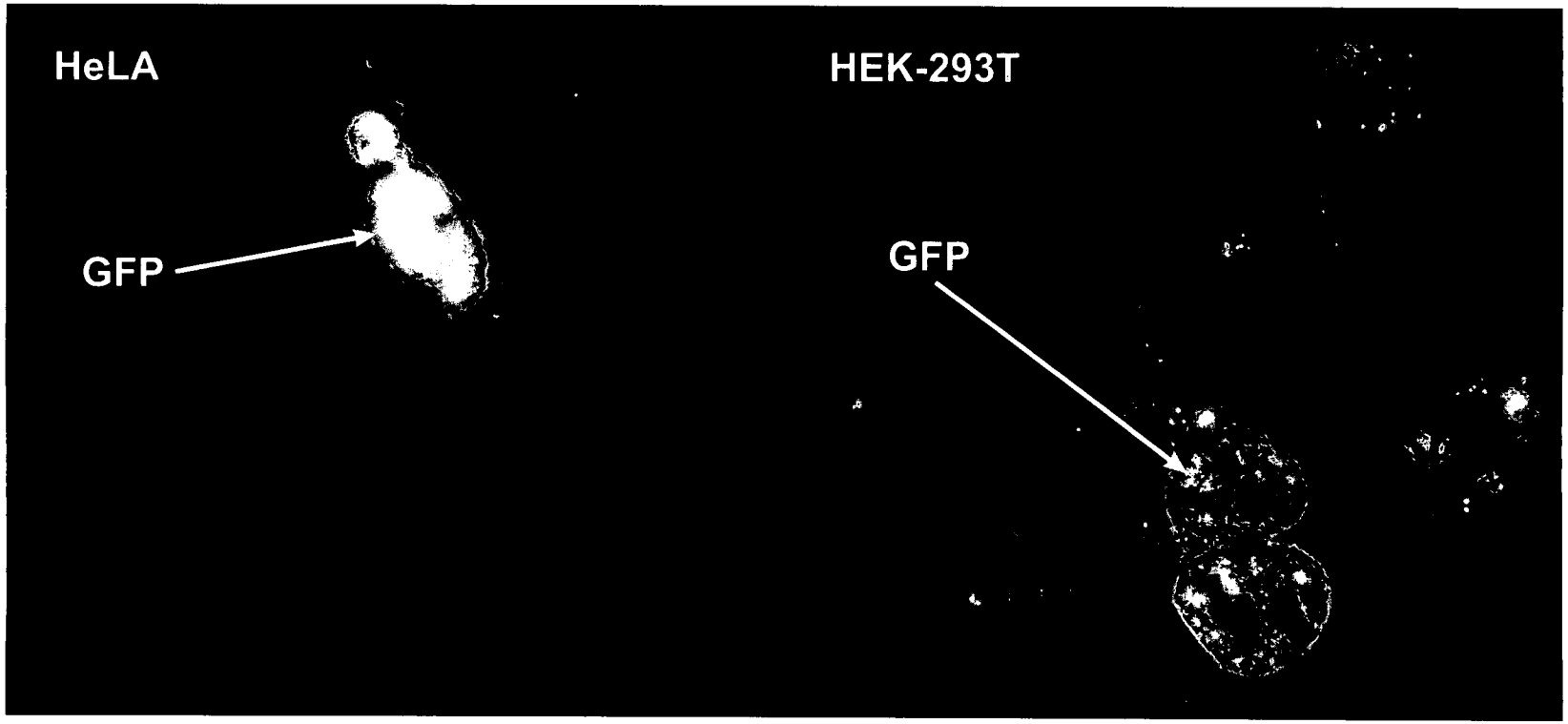


FIG. 26.

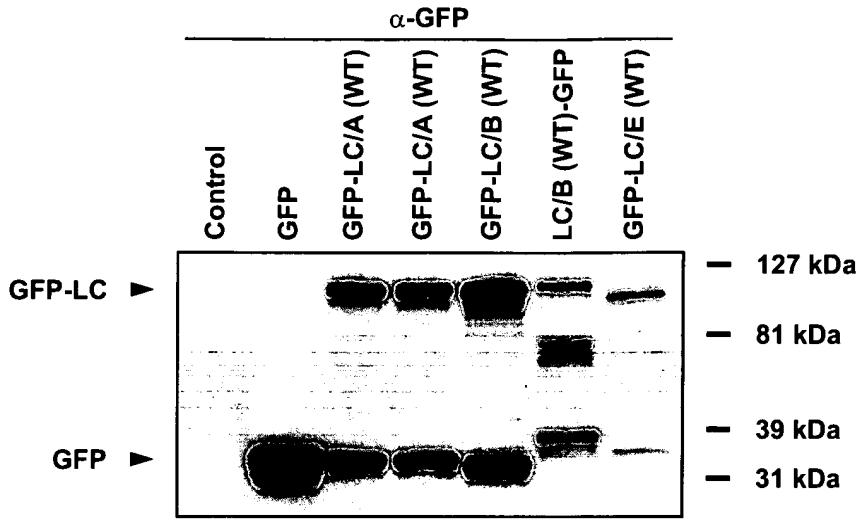

α-GFP
Control
GFP
GFP-LC/A (WT)
GFP-LC/A (WT)
GFP-LC/B (WT)
LC/B (WT)-GFP
GFP-LC/E (WT)
GFP-LC
GFP
127 kDa
81 kDa
39 kDa
31 kDa

FIG. 27.

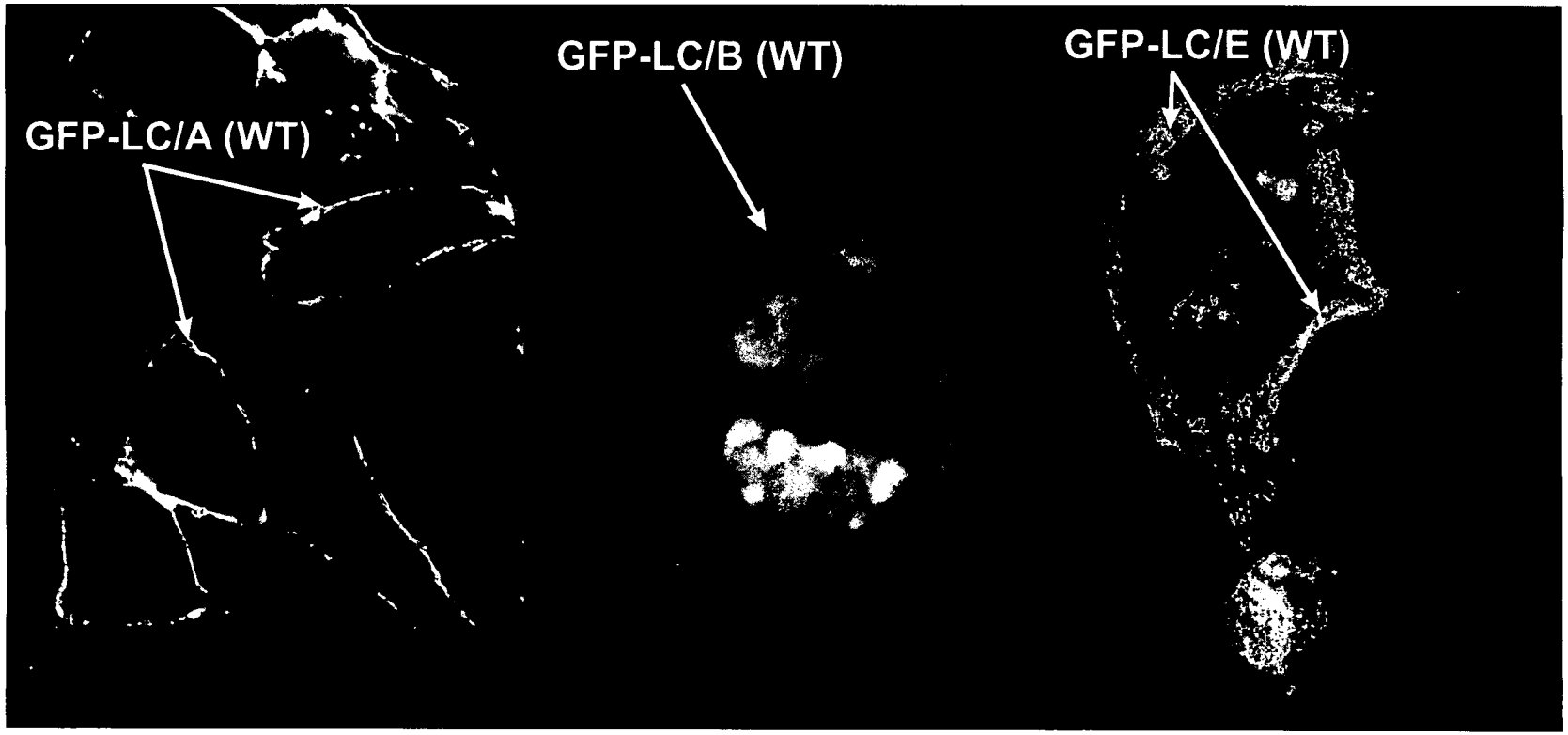


FIG. 28.

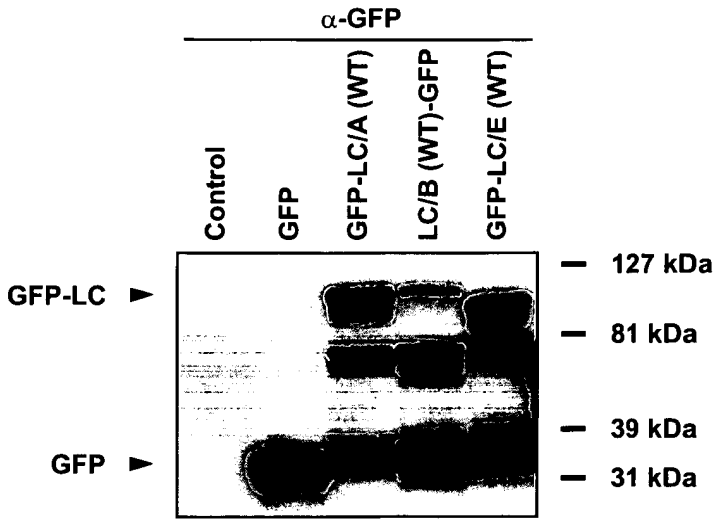

α-GFP
Control
GFP
GFP-LC/A (WT)
LC/B (WT)-GFP
GFP-LC/E (WT)
GFP-LC
GFP
127 kDa
81 kDa
39 kDa
31 kDa

FIG. 29.

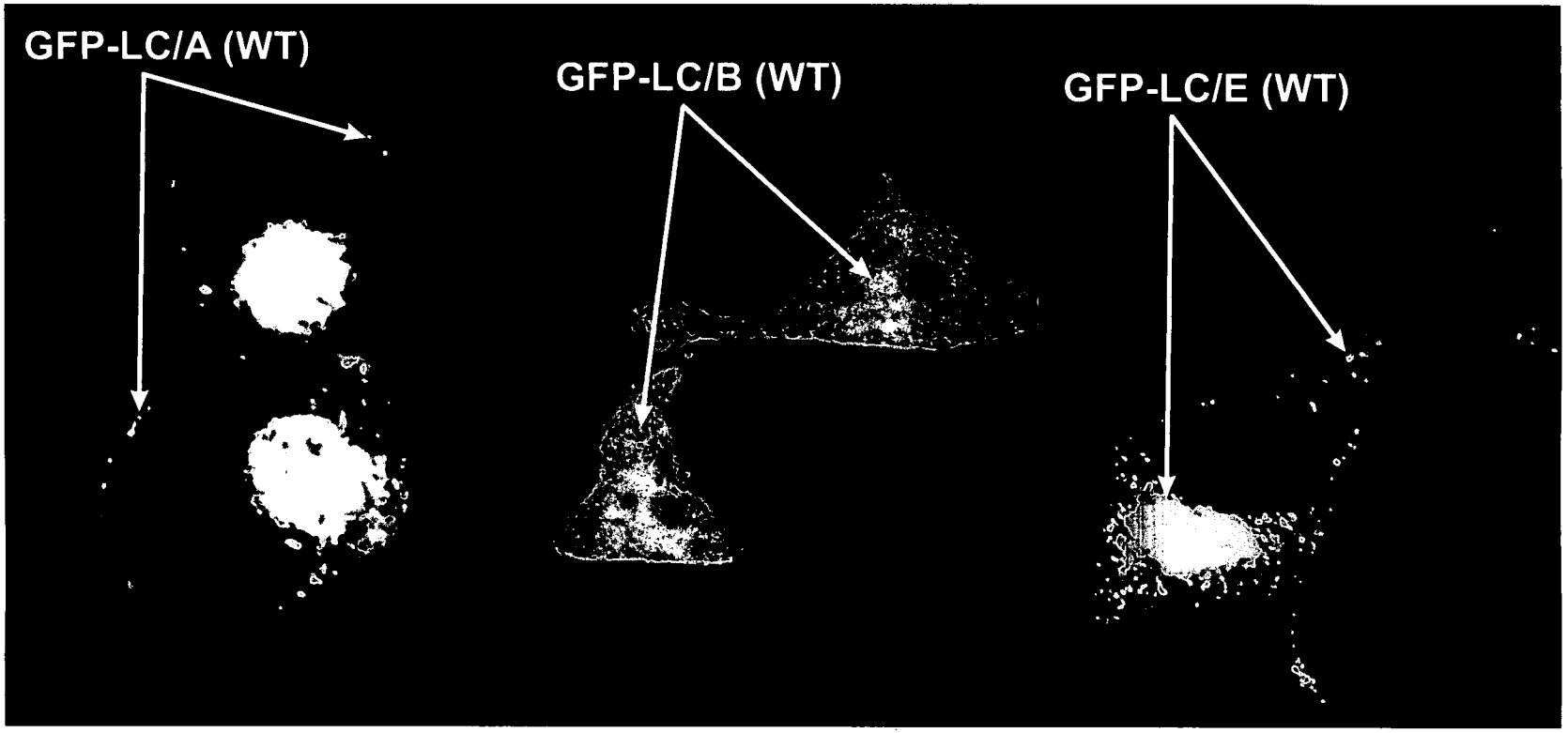


FIG. 30A.

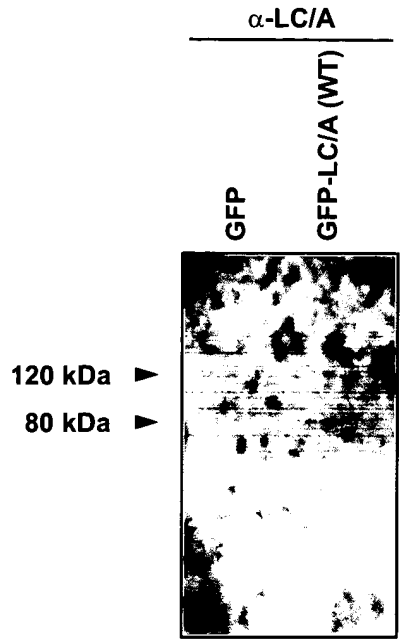


FIG. 30B.

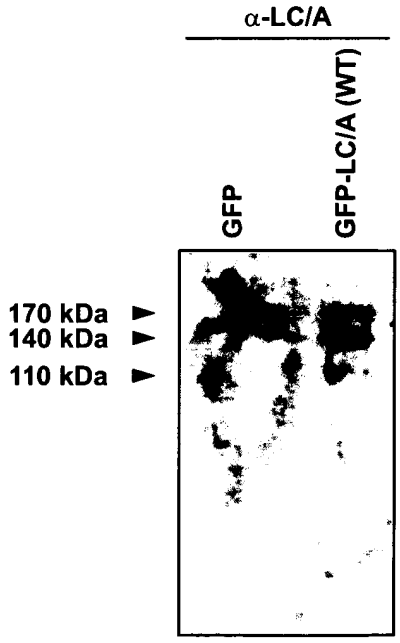


FIG. 31A.

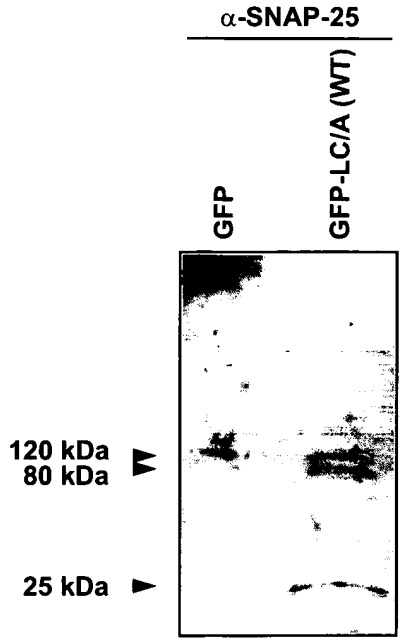


FIG. 31B.

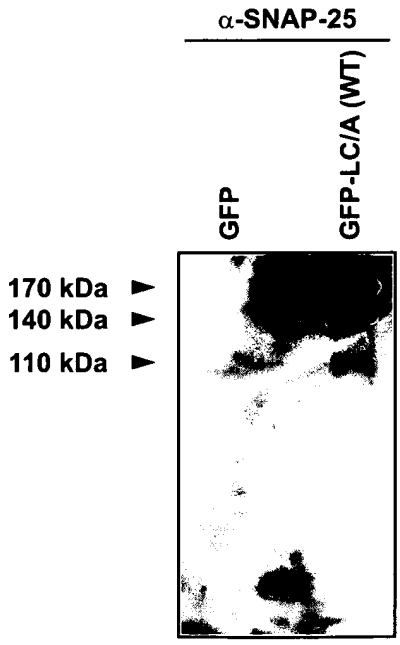


FIG. 32.

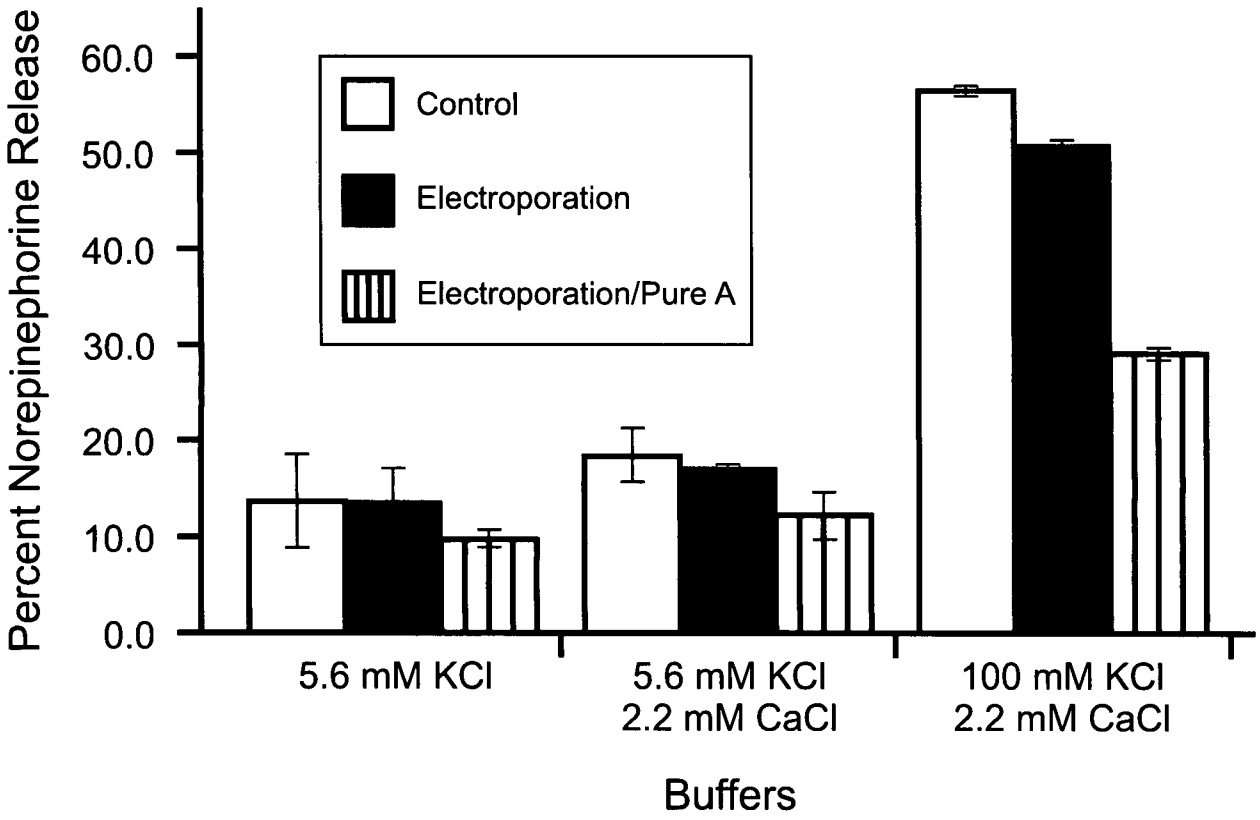

Percent Norepinephorine Release
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Control
Electroporation
Electroporation/Pure A
5.6 mM KCl
5.6 mM KCl
2.2 mM CaCl
100 mM KCl
2.2 mM CaCl
Buffers

FIG. 33.

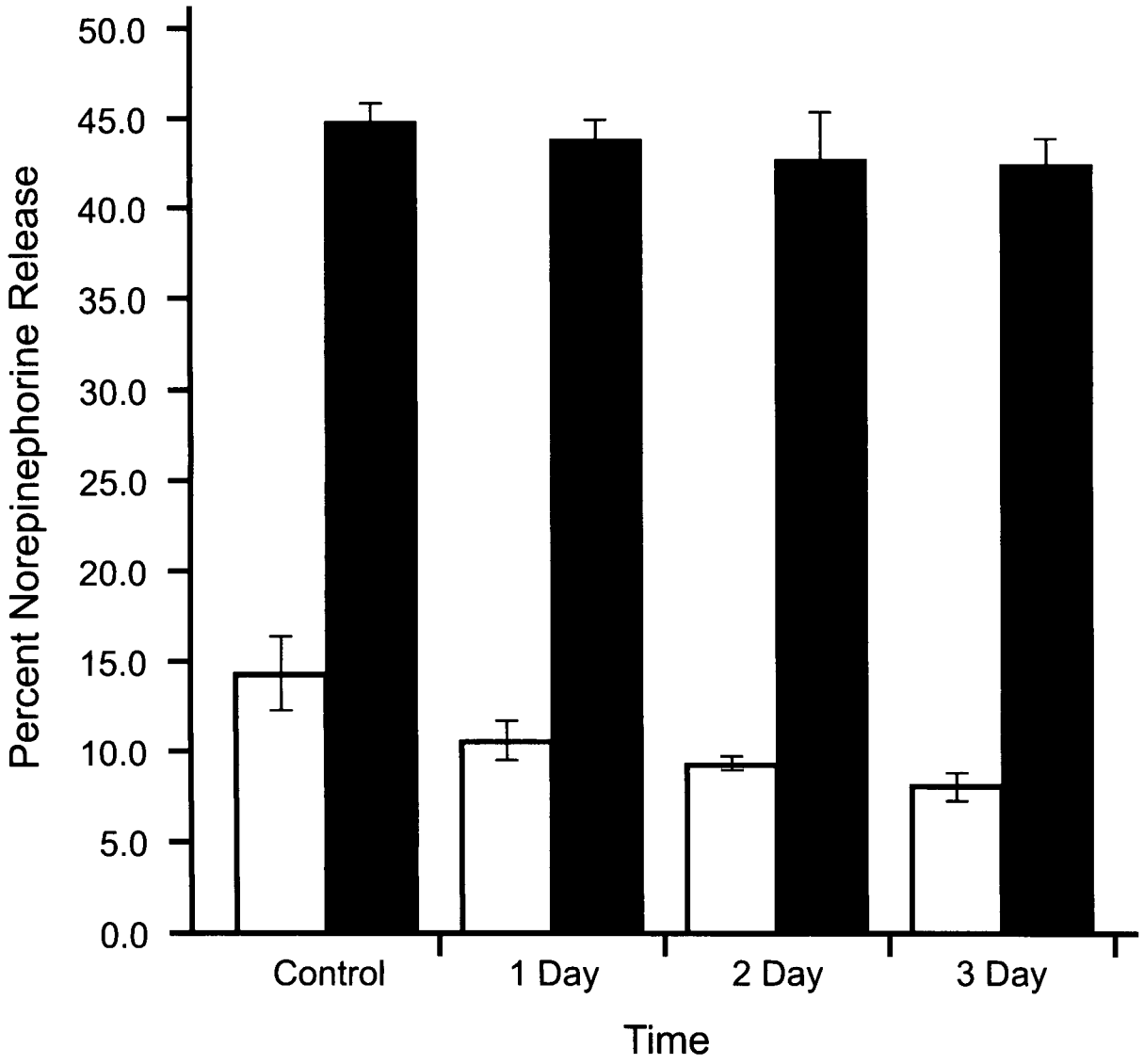

Percent Norepinephorine Release
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Control
1 Day
2 Day
3 Day
Time

FIG. 34.

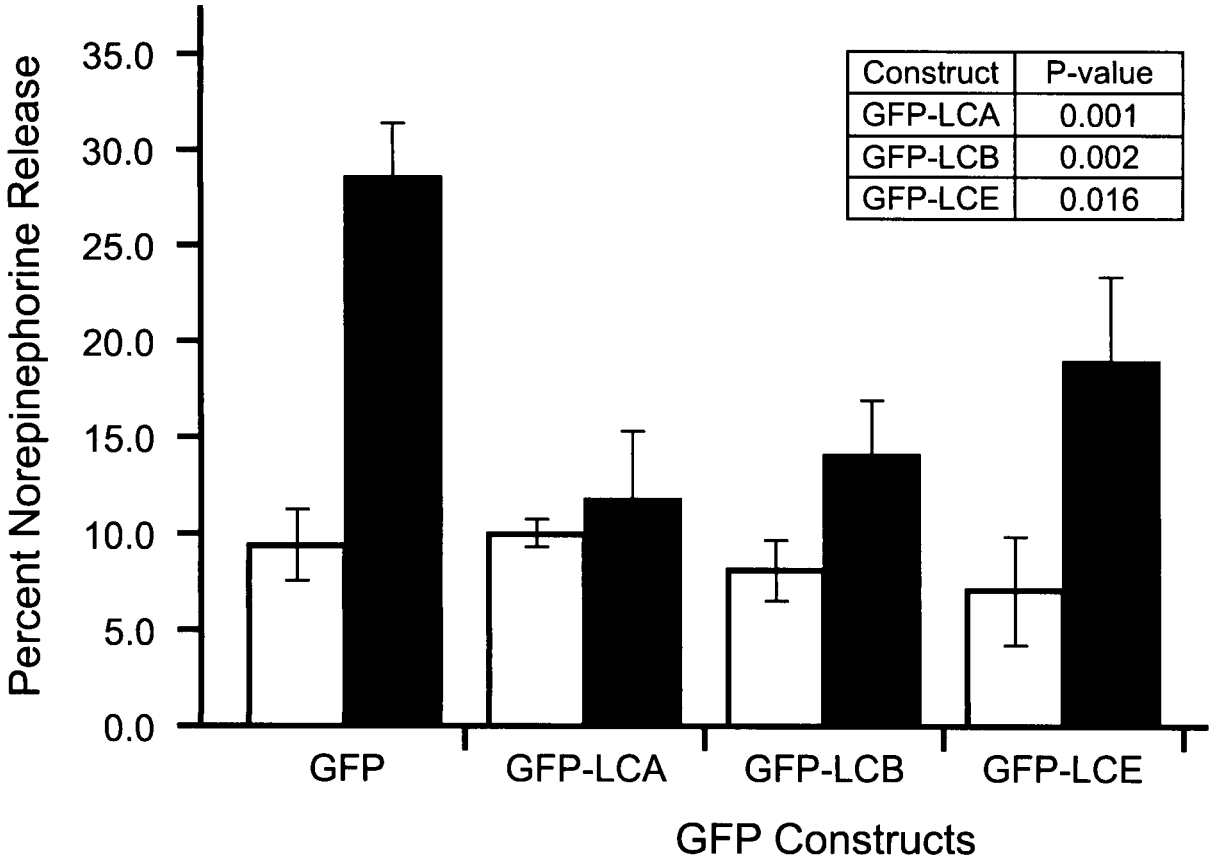

Percent Norepinephorine Release
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
GFP
GFP-LCA
GFP-LCB
GFP-LCE
GFP Constructs
Construct
P-value
GFP-LCA
0.001
GFP-LCB
0.002
GFP-LCE
0.016

FIG. 35.

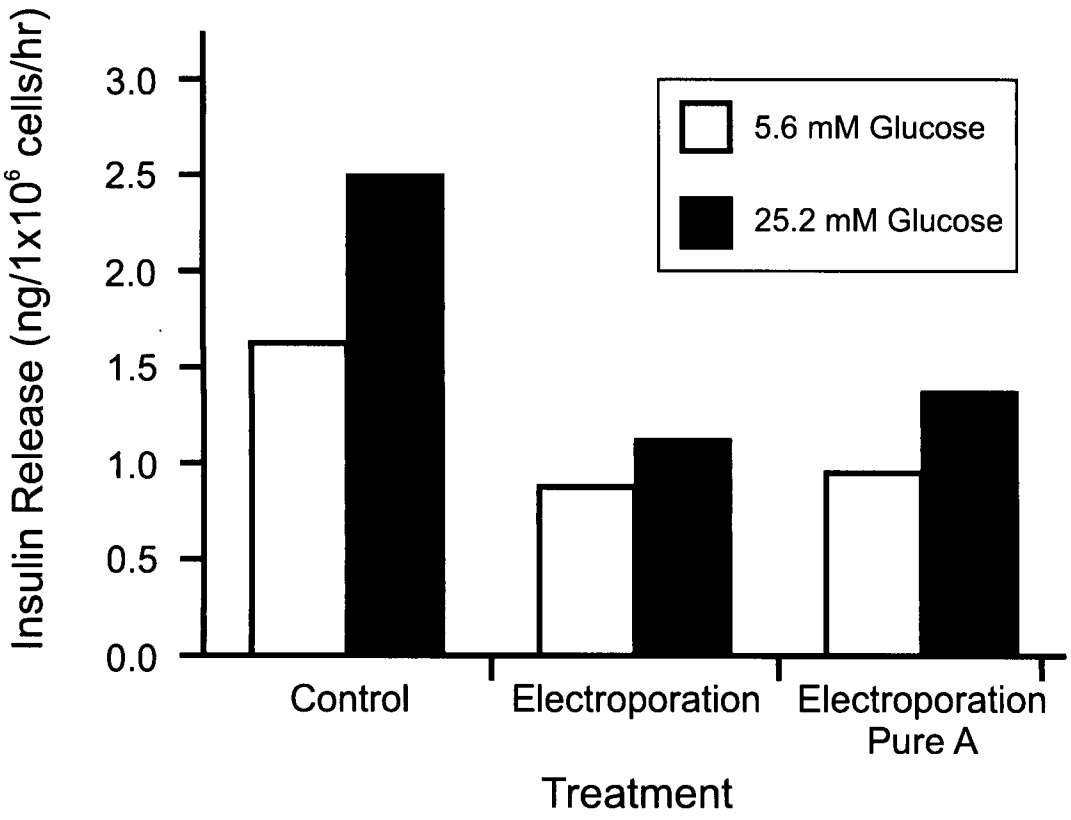

Insulin Release (ng/1x10^6 cells/hr)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
5.6 mM Glucose
25.2 mM Glucose
Control
Electroporation
Electroporation Pure A
Treatment

FIG. 36.

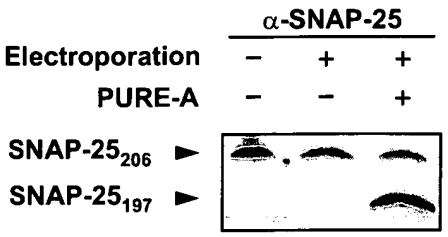
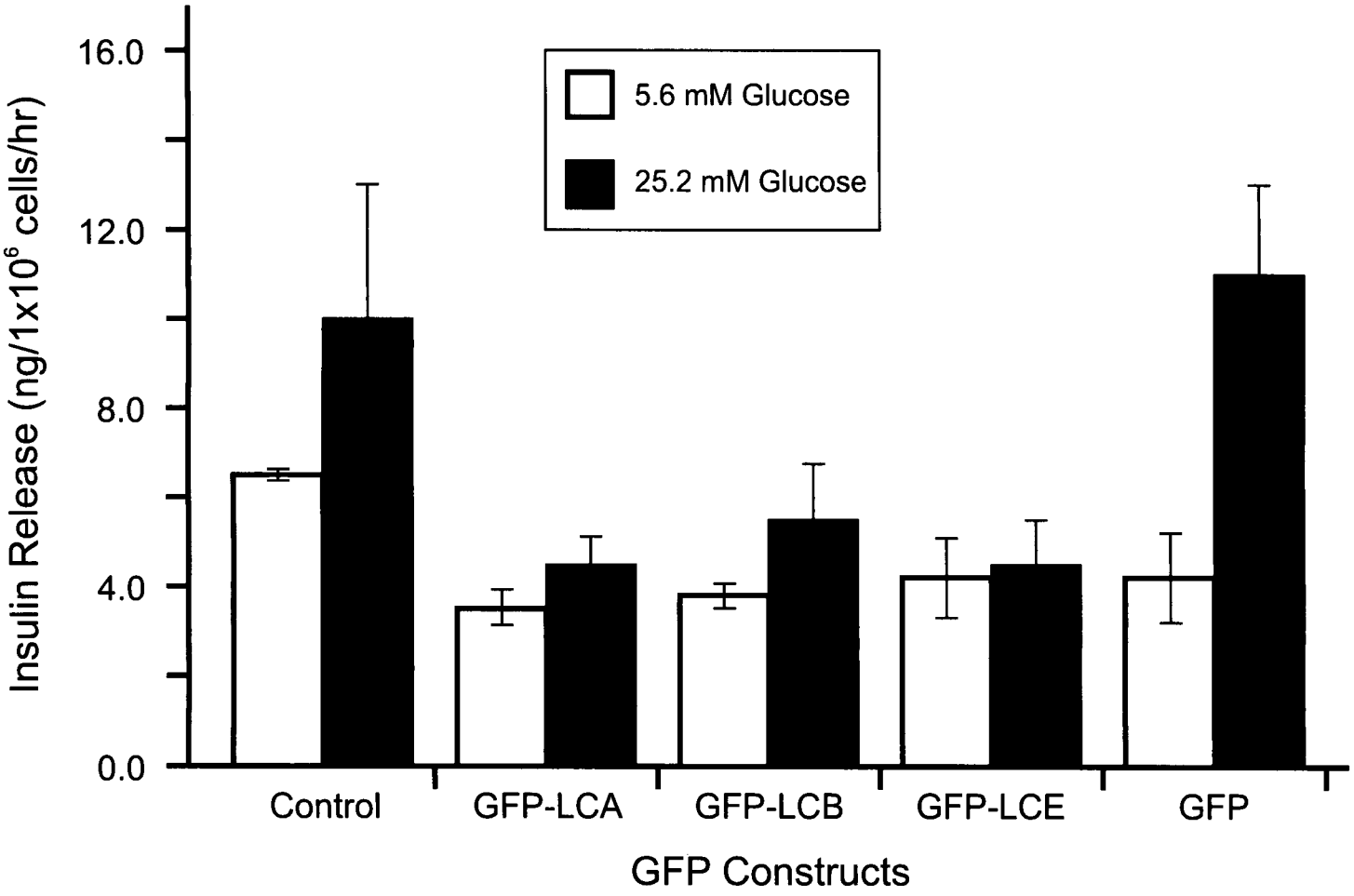
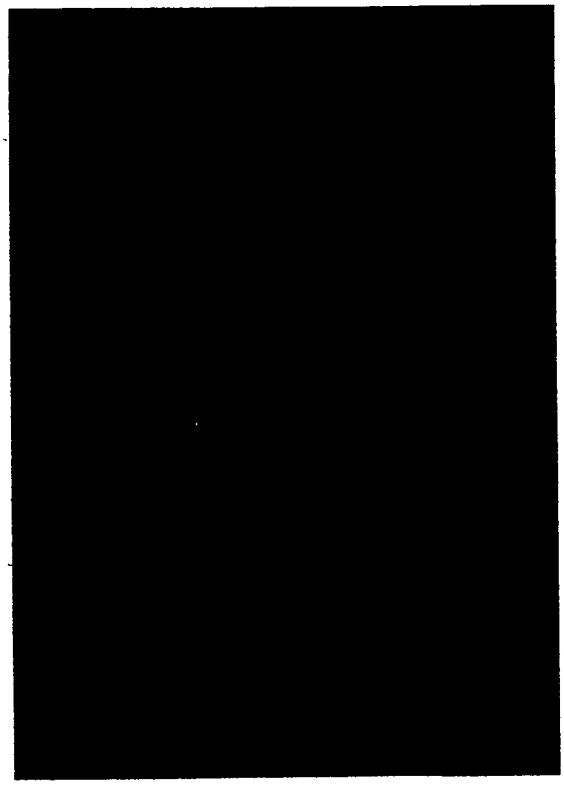


FIG. 37.

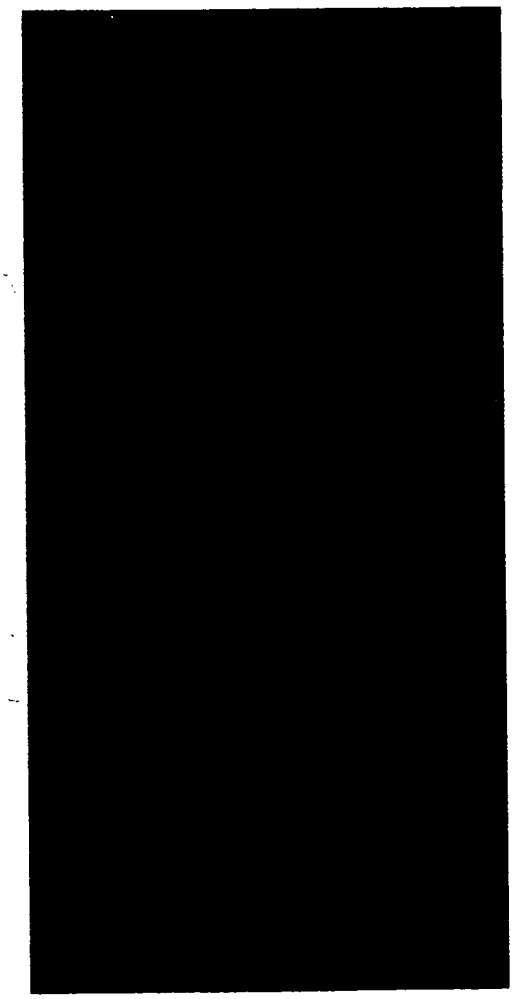

16.0
12.0
8.0
4.0
0.0
Insulin Release (ng/1x10^6 cells/hr)
5.6 mM Glucose
25.2 mM Glucose
Control
GFP-LCA
GFP-LCB
GFP-LCE
GFP
GFP Constructs

USPTO MAIL ROOM
FEB 01 2007
PATENT & TRADEMARK OFFICE



GFP-LCA (wt)

FIG. 1



GFP-TLCA

FIG. 2

```
  1  PFVNKQFNYK DPVNGVDIAY IKIPNVGQMQ PVKAFKIHNK IWVIPERDTF
 51  TNPEEGDLNP PPEAKQVPVS YYDSTYLSTD NEKDNYLKGV TKLFERIYST
101  DLGRMLLTSI VRGIPFWGGS TIDTELKVID TNCINVIQPD GSYRSEELNL
151  VIIGPSADII QFECKSFGHE VLNLTRNGYG STQYIRFSPD FTFGFEESLE
201  VDTNPLLGAG KFATDPAVTL AHELIHAGHR LYGIAINPNR VFKVNTNAYY
251  EMSGLEVSFE ELRTFGGHDA KFIDSLQENE FRLYYYNKFK DIASTLNKAK
301  SIVGTTASLQ YMKNVFKEKY LLSEDTSGKF SVDKLKFDKL YKMLTEIYTE
351  DNFVKFFKVL NRKTYLNFDK AVFKINIVPK VNYTIYDGFN LRNTNLAANF
401  NGQNTEINNM NFTKLKNFTG LFEFYKLLCV RGIITSK
```

FIG. 3

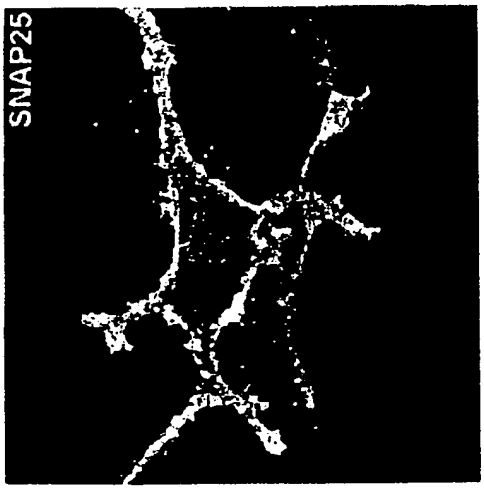


FIG. 5

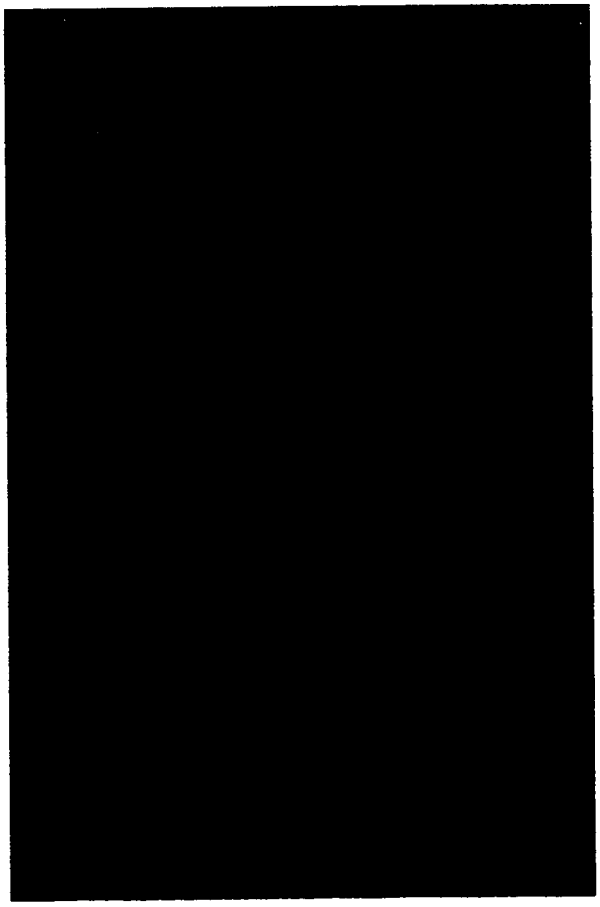
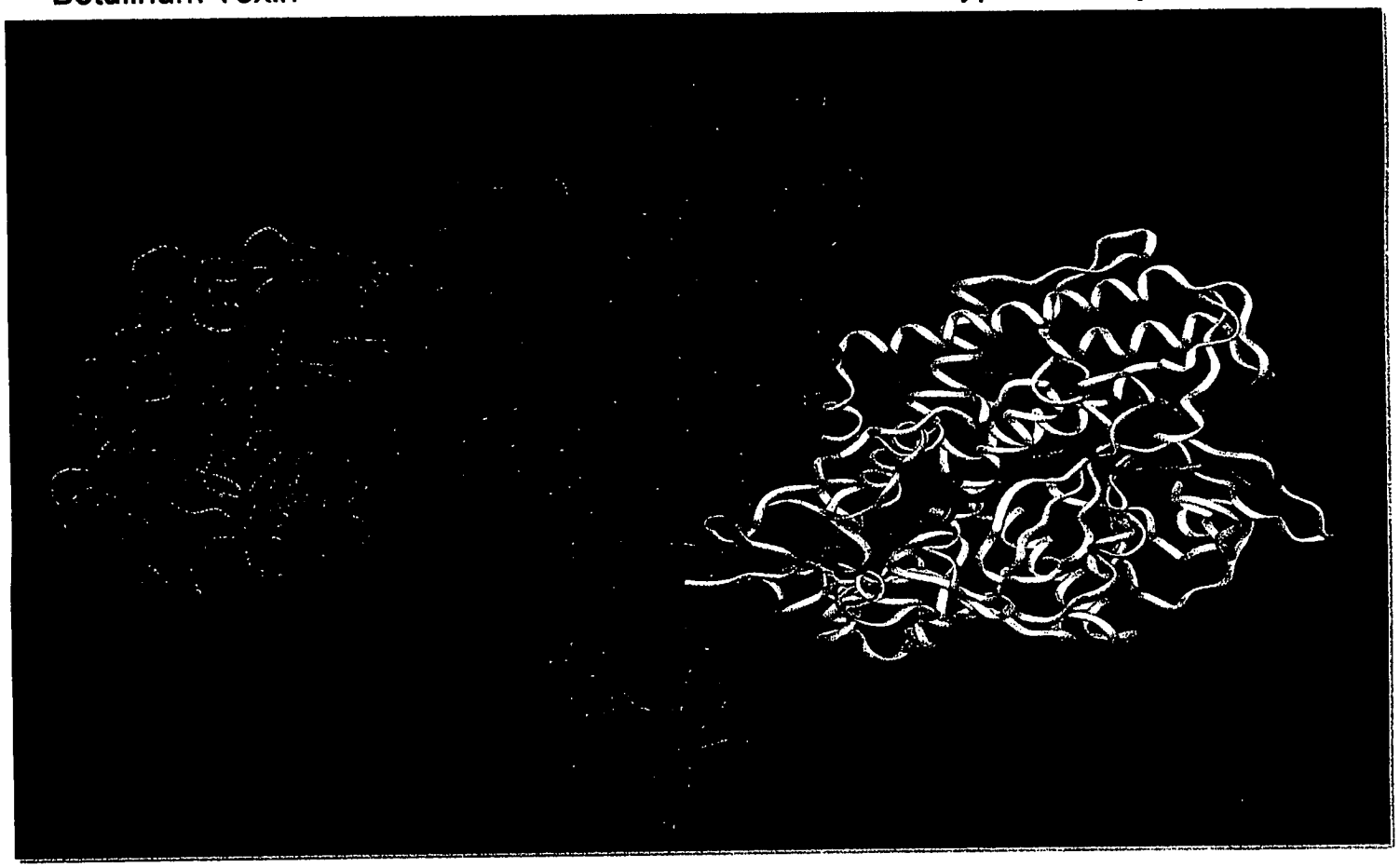


FIG. 4

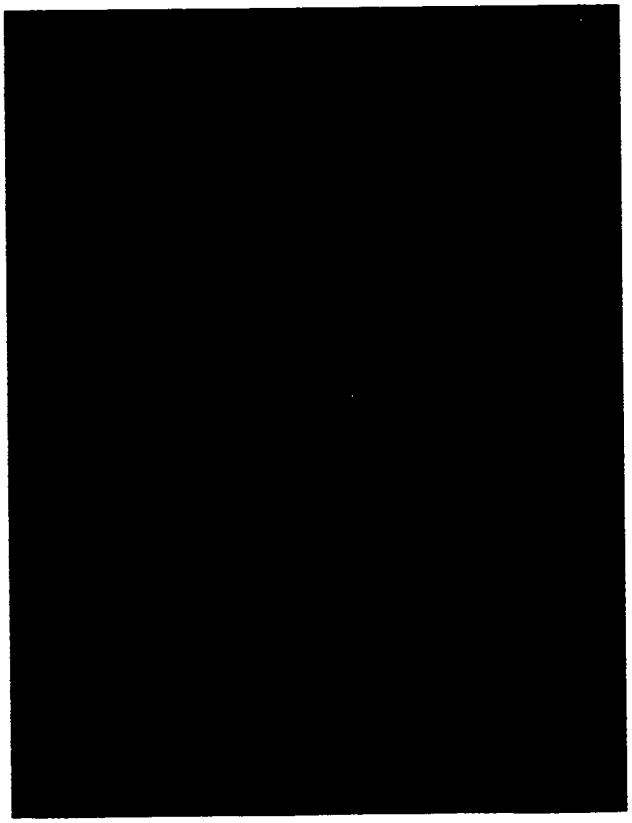
Botulinum Toxin

Type A X-Ray Structure



Yellow = protease domain
Green = binding domain
Red = translocational domain

FIG. 6



GFP-LCB (wt)

FIG. 7

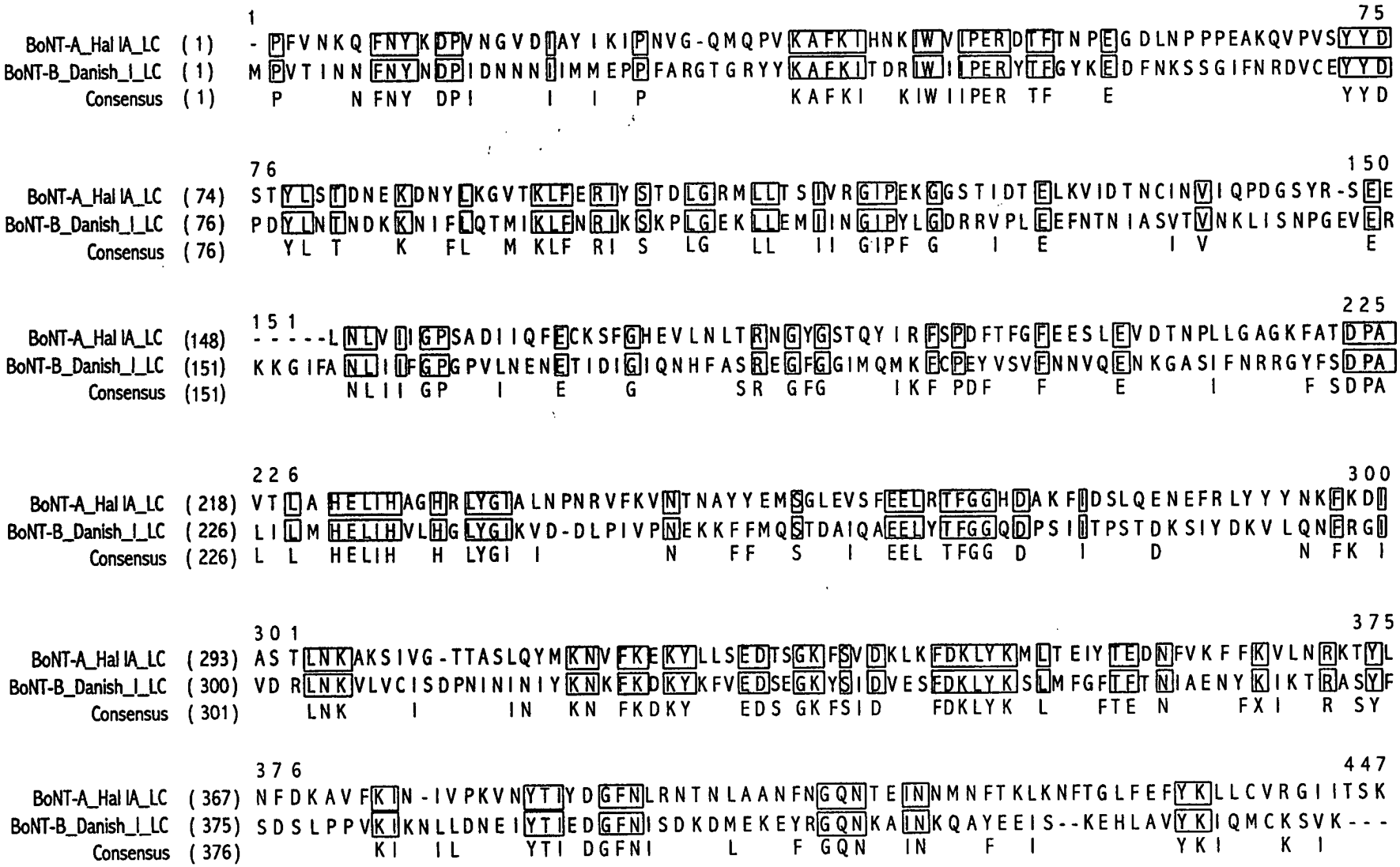

1 75
BoNT-A_Hal IA_LC (1) - PFVNKQFNYKDPVNGVDIAYIKIPNVG-QMQPVKAFKIHNKIWVIPERDTFTNPEGDLNPPPEAKQVPVSYYD
BoNT-B_Danish_I_LC (1) MPVTINNFNYNDPIDNNNIIMMEPPFARGTGRYYKAFKITDRIWIIPERYTFGYKEDFNKSSGIFNRDVCEYYD
Consensus (1) P N FNY DPI I I P KAFKI KIWIIPER TF E YYD
76 150
BoNT-A_Hal IA_LC (74) STYLSTDNEKDNYLKGVTKLFERIYSTDLGRMLLTSIVRGIPEKGGSTIDTELKVIDTNCINVIQPDGSYR-SEE
BoNT-B_Danish_I_LC (76) PDYLNTNDKKNIFLQTMIKLFNRIKSKPLGEKLLEMIINGIPYLGDRRVPLEEFNTNIASVTVNKLISNPGEVER
Consensus (76) YL T K FL M KLF RI S LG LL II GIPF G I E I V E
151 225
BoNT-A_Hal IA_LC (148) -----LNLVIIGPSADIIQFECKSFGHEVLNLTRNGYGSTQYIRFSPDFTFGFEESLEVDTNPLLGAGKFATDPA
BoNT-B_Danish_I_LC (151) KKGIFANLIIFGPGPVLNENETIDIGIQNHFASREGFGGIMQMKFCPEYVSVFNNVQENKGASIFNRRGYFSDPA
Consensus (151) NLII GP I E G SR GFG IKF PDF F E I F SDPA
226 300
BoNT-A_Hal IA_LC (218) VTLAHELIHAGHRLYGIALNPNRVFKVNTNAYYEMSGLEVSFEELRTFGGHDAKFIDSLQENEFRLYYYNKFKDI
BoNT-B_Danish_I_LC (226) LILMHELIHVLHGLYGIKVD-DLPIVPNEKKFFMQSTDAIQAEELYTFGGQDPSIITPSTDKSIYDKVLQNFRGI
Consensus (226) L L HELIH H LYGI I N FF S I EEL TFGG D I D N FK I
301 375
BoNT-A_Hal IA_LC (293) ASTLNKAKSIVG-TTASLQYMKNVFKEKYLLSEDTSGKFSVDKLKFDKLYKMLTEIYTEDNFVKFFKVLNRKTYL
BoNT-B_Danish_I_LC (300) VDRLNKVLVCISDPNININIYKNKFKDKYKFVEDSEGKYSIDVESFDKLYKSLMFGFTFTNIAENYKIKTRASYF
Consensus (301) LNK I IN KN FKDKY EDS GK FSID FDKLYK L FTE N FXI R SY
376 447
BoNT-A_Hal IA_LC (367) NFDKAVFKIN-IVPKVNYTIYDGFNLRNTNLAANFNGQNTEINNMNFTKLKNFTGLFEFYKLLCVRGIITSK
BoNT-B_Danish_I_LC (375) SDSLPPVKIKNLLDNEIYTIEDGFNISDKDMEKEYRGQNKAINKQAYEEIS--KEHLAVYKIQMCKSVK---
Consensus (376) KI IL YTI DGFNI L F GQN IN F I YKI K I

FIG. 8

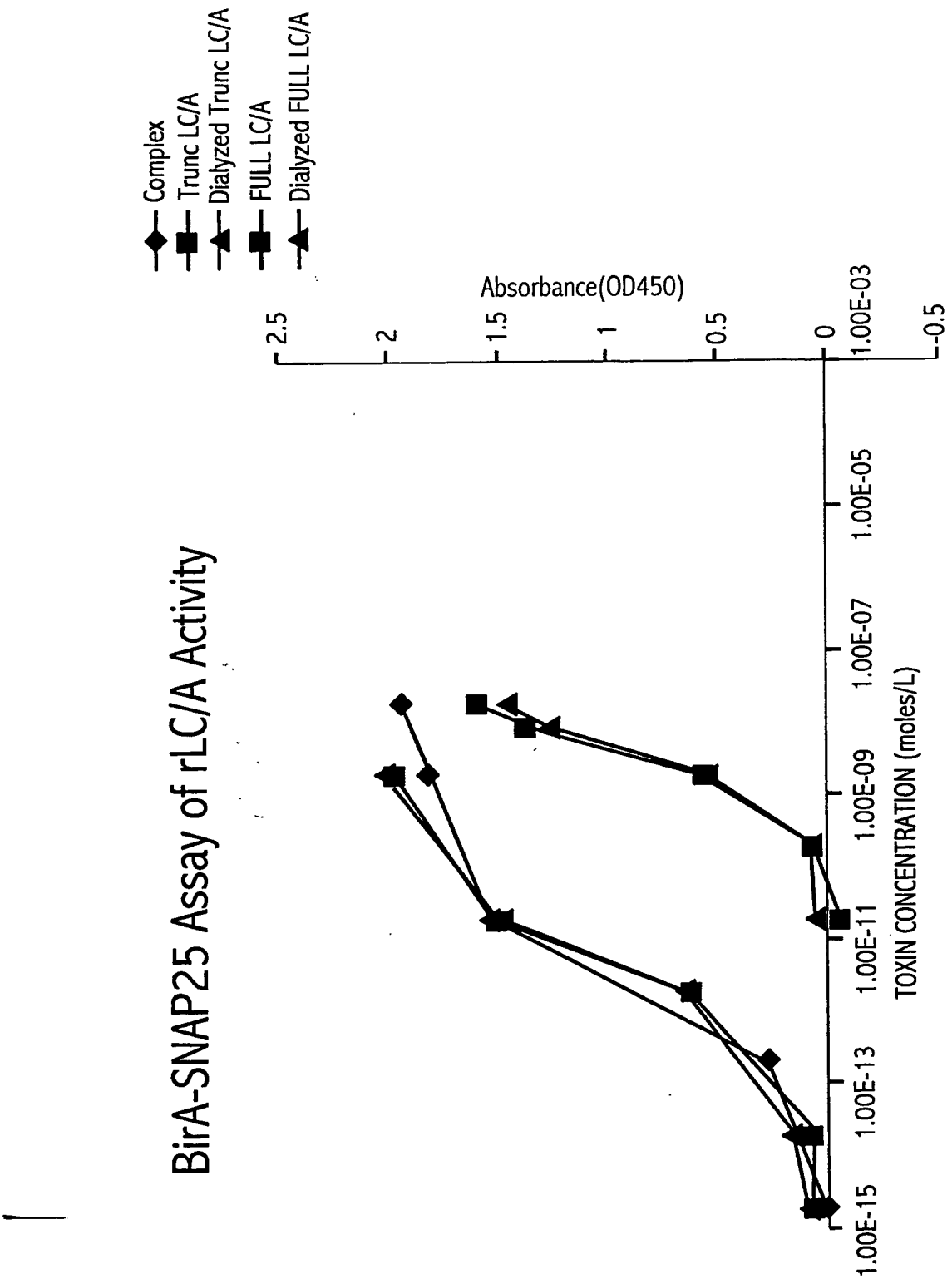
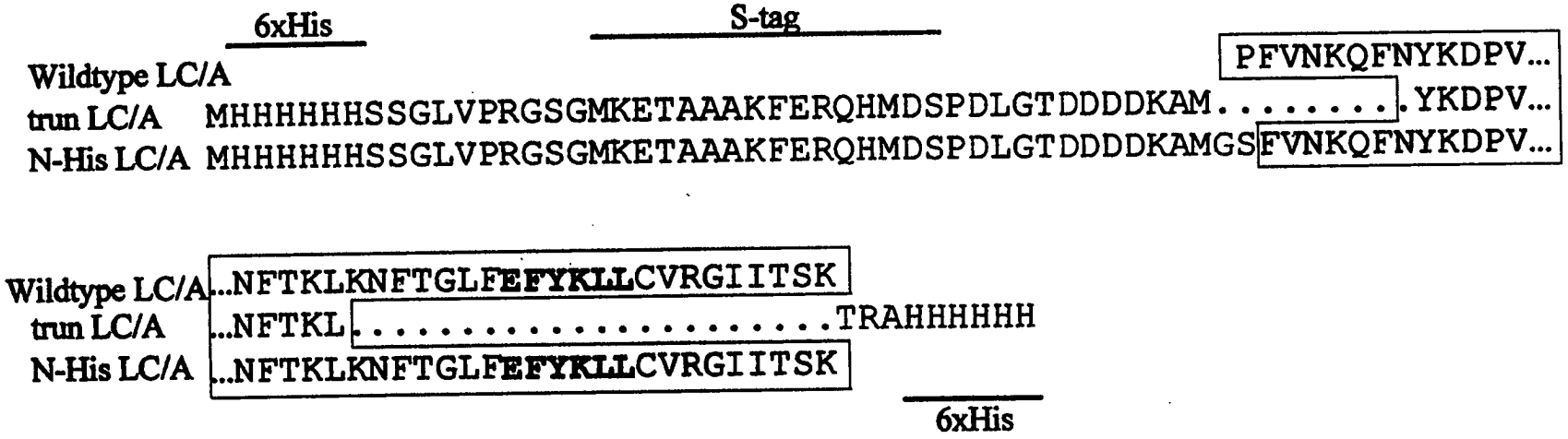

BirA-SNAP25 Assay of rLC/A Activity
Complex
Trunc LC/A
Dialyzed Trunc LC/A
FULL LC/A
Dialyzed FULL LC/A
Absorbance(OD450)
2.5
2
1.5
1
0.5
0
-0.5
1.00E-15
1.00E-13
1.00E-11
1.00E-09
1.00E-07
1.00E-05
1.00E-03
TOXIN CONCENTRATION (moles/L)

FIG. 9

Comparison of LC/A constructs expressed from E. coli for in vitro analysis



- Truncated LC construct published
  - Kadkhodayan, S. et al. Prot. Exp. Purif. 2000, 19, 125-130
  - Crystal structure reported at IBRCC in Oct. 2000

FIG. 10

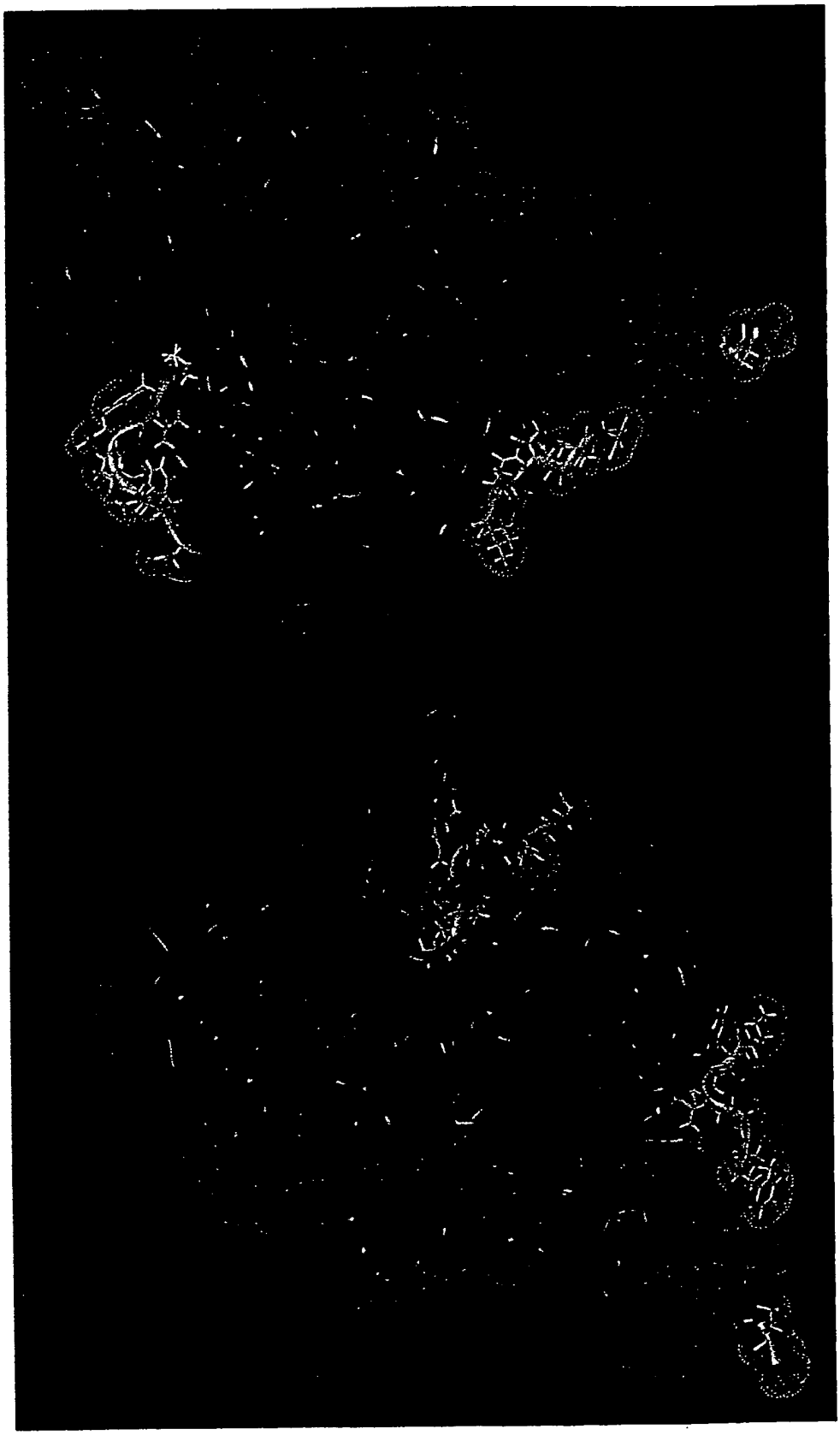


FIG. 11

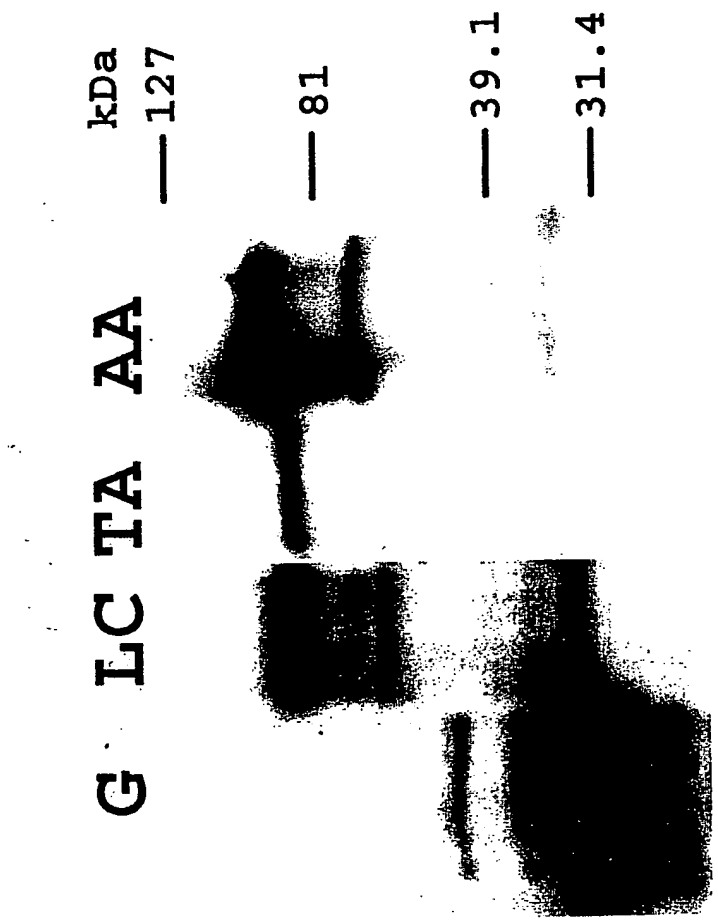


FIG. 12

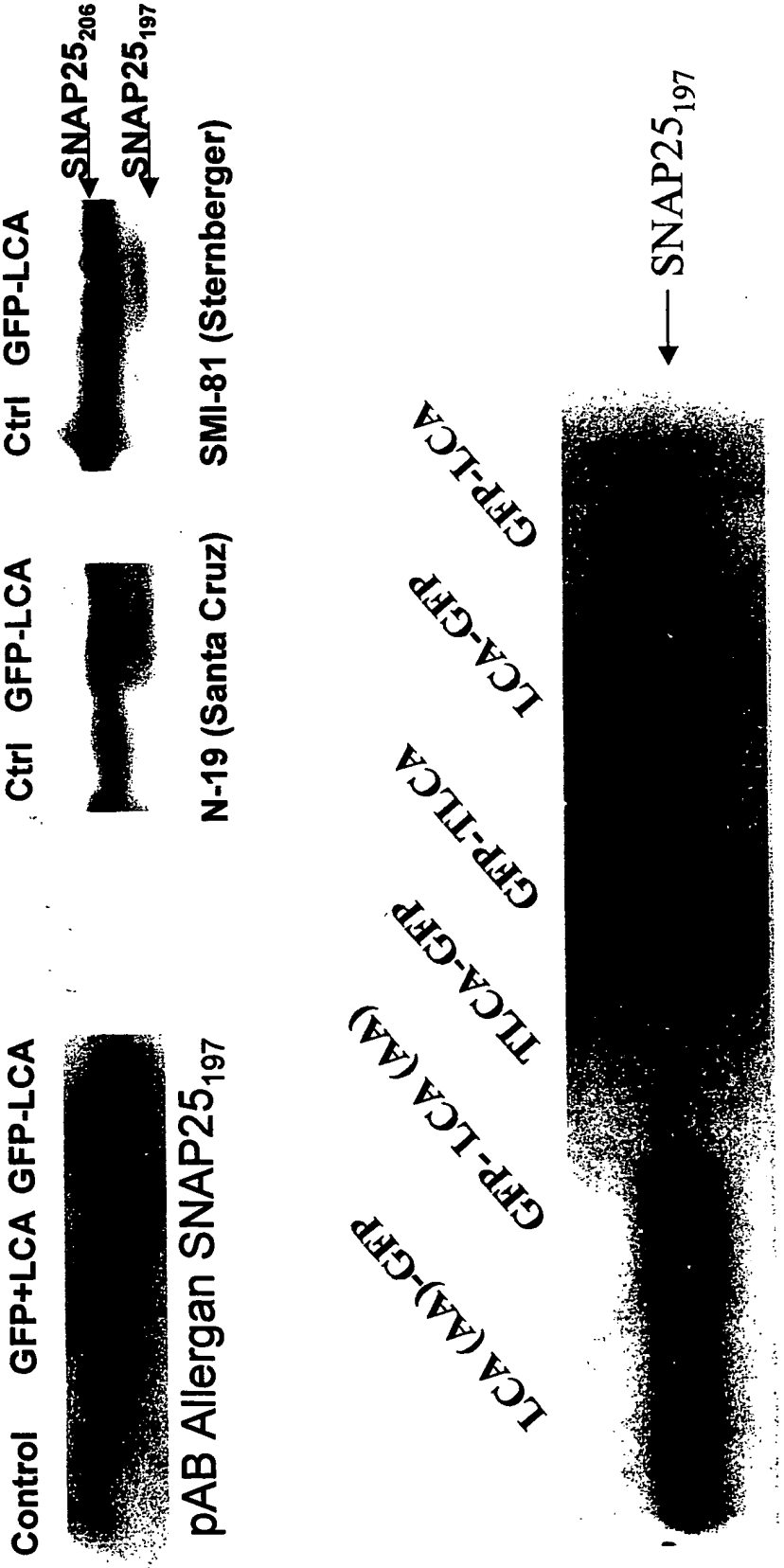

Control
GFP+LCA
GFP-LCA
pAB Allergan SNAP25197
Ctrl
GFP-LCA
N-19 (Santa Cruz)
Ctrl
GFP-LCA
SMI-81 (Sternberger)
SNAP25206
SNAP25197
LCA (AA)-GFP
GFP-LCA (AA)
TLCA-GFP
GFP-TLCA
LCA-GFP
GFP-LCA
SNAP25197

FIG. 13

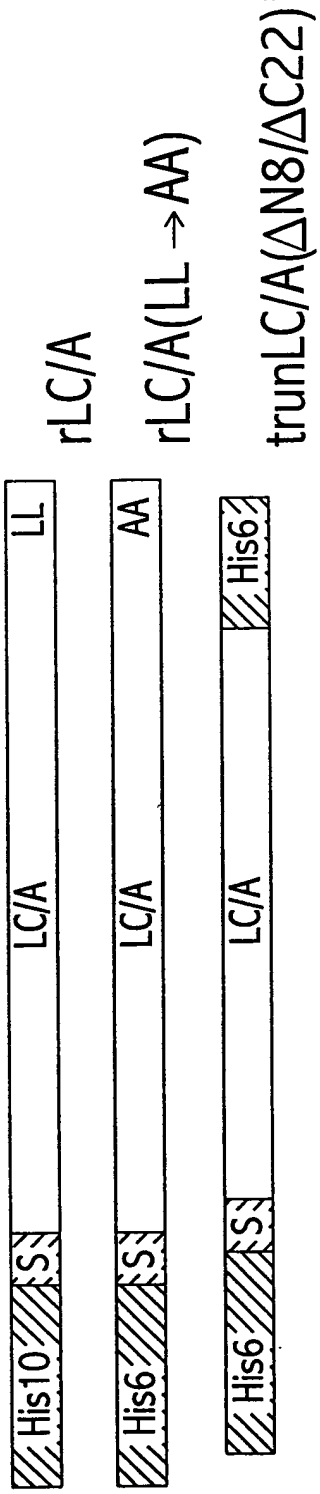

rLC/A
rLC/A(LL → AA)
trunLC/A(ΔN8/ΔC22)*
His10
S
LC/A
LL
His6
S
LC/A
AA
His6
S
LC/A
His6

FIG. 14

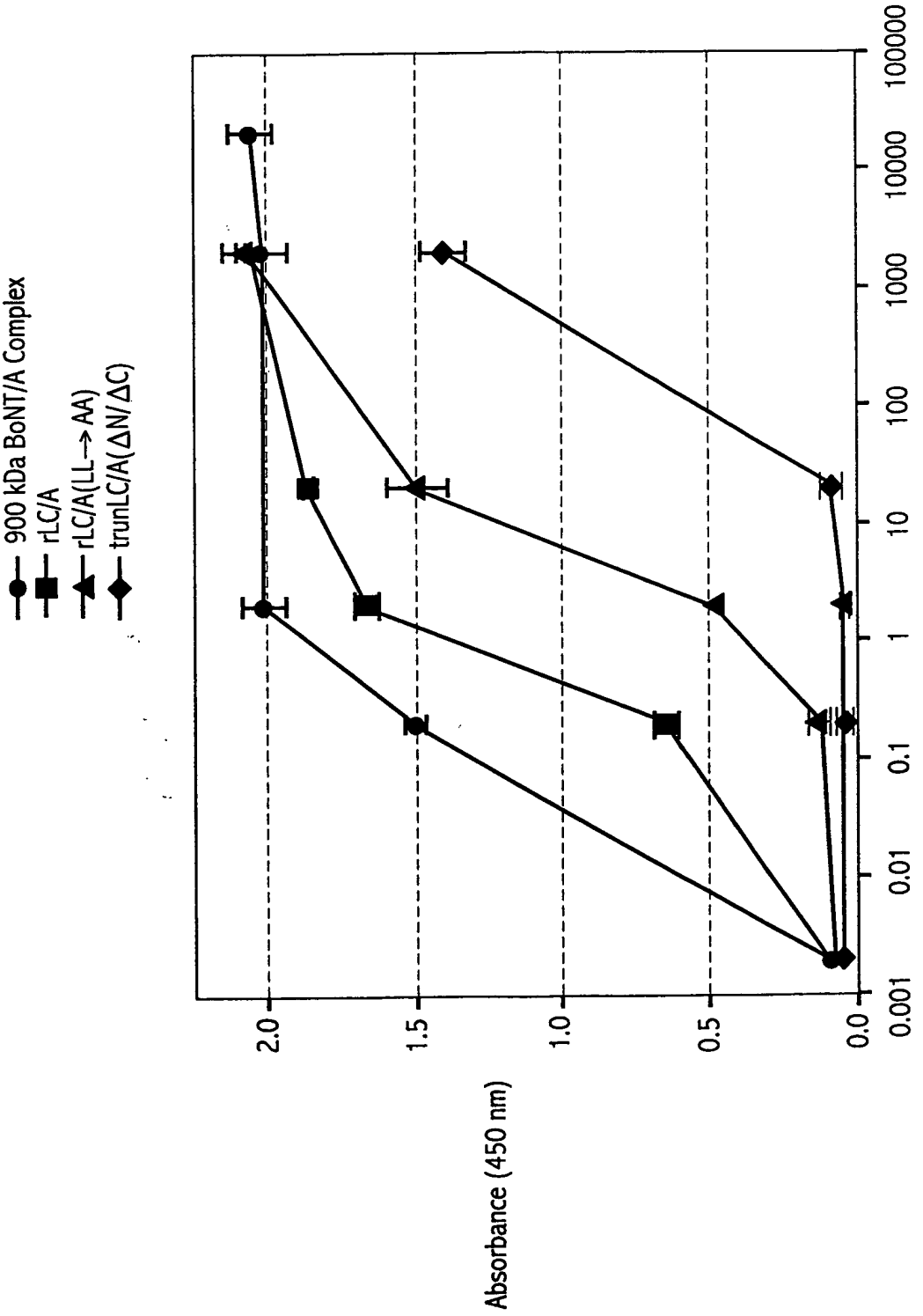

900 kDa BoNT/A Complex
rLC/A
rLC/A(LL→AA)
trunLC/A(ΔN/ΔC)
Absorbance (450 nm)
2.0
1.5
1.0
0.5
0.0
0.001
0.01
0.1
1
10
100
1000
10000
100000
Concentration (pM)

FIG. 15

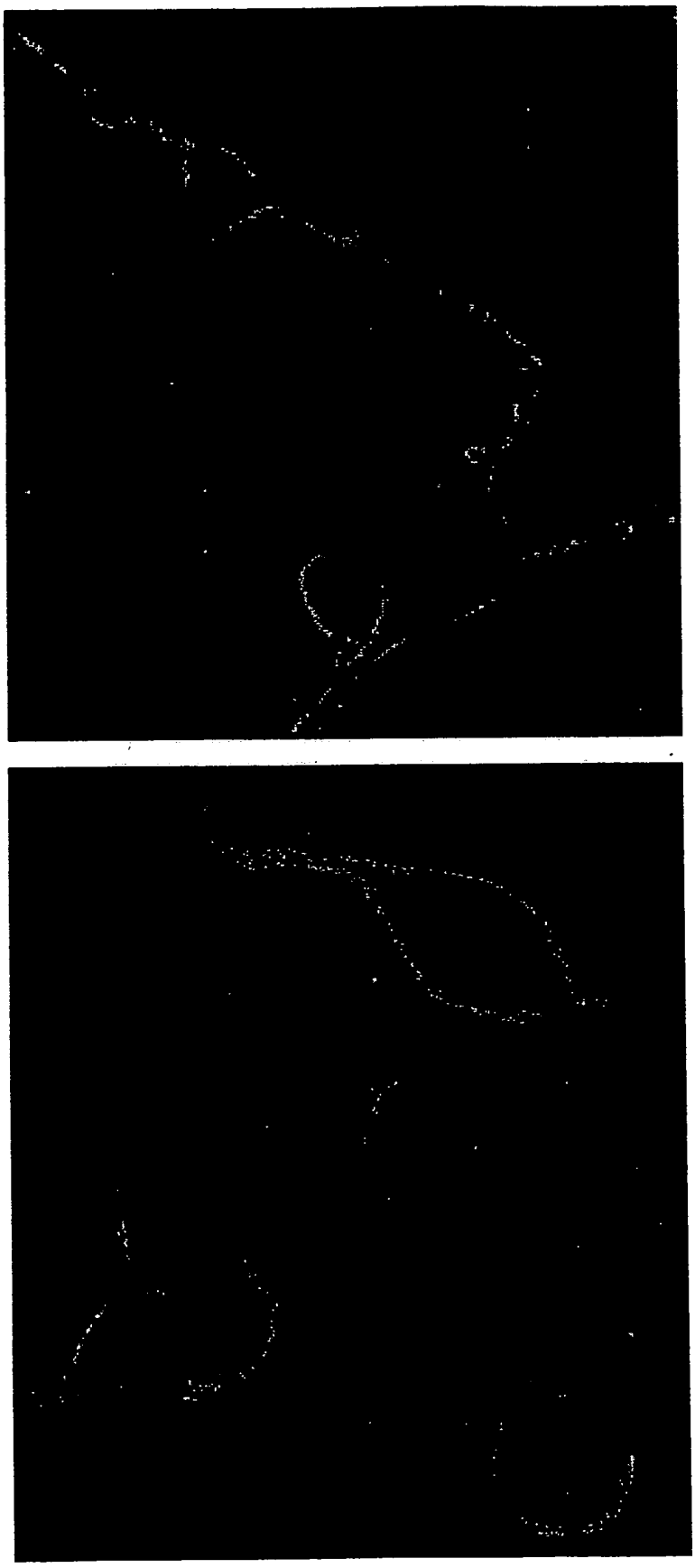


FIG. 16

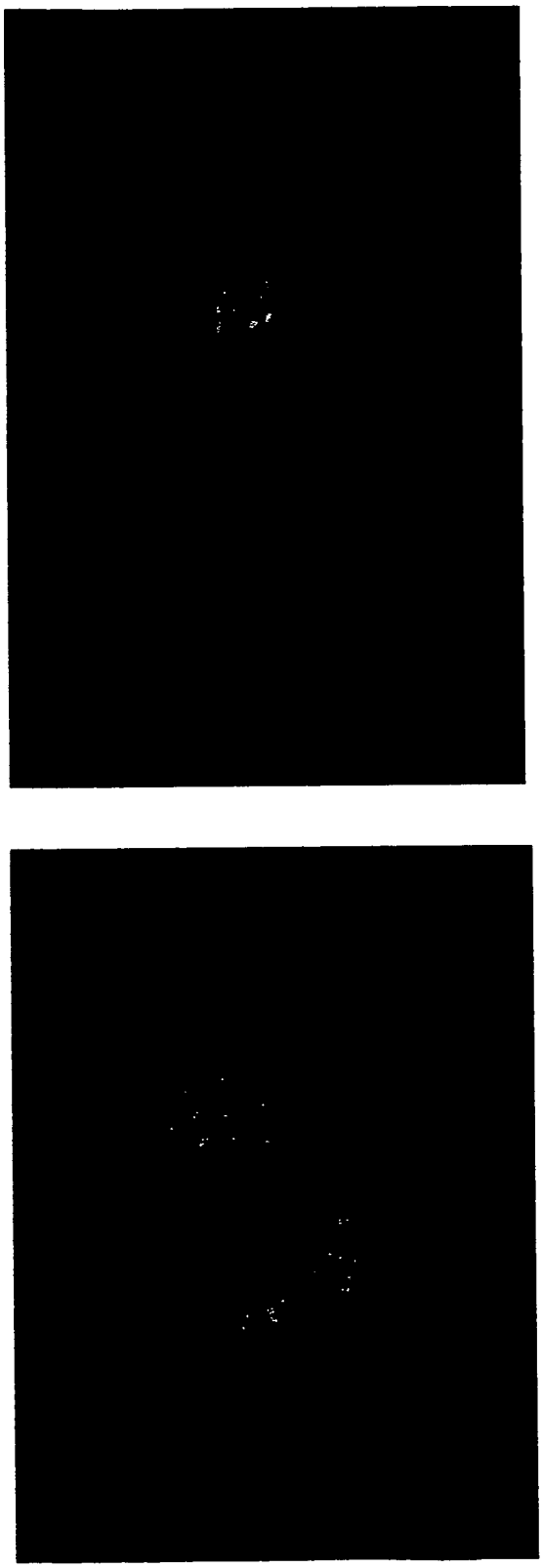


FIG. 17

FIG. 18

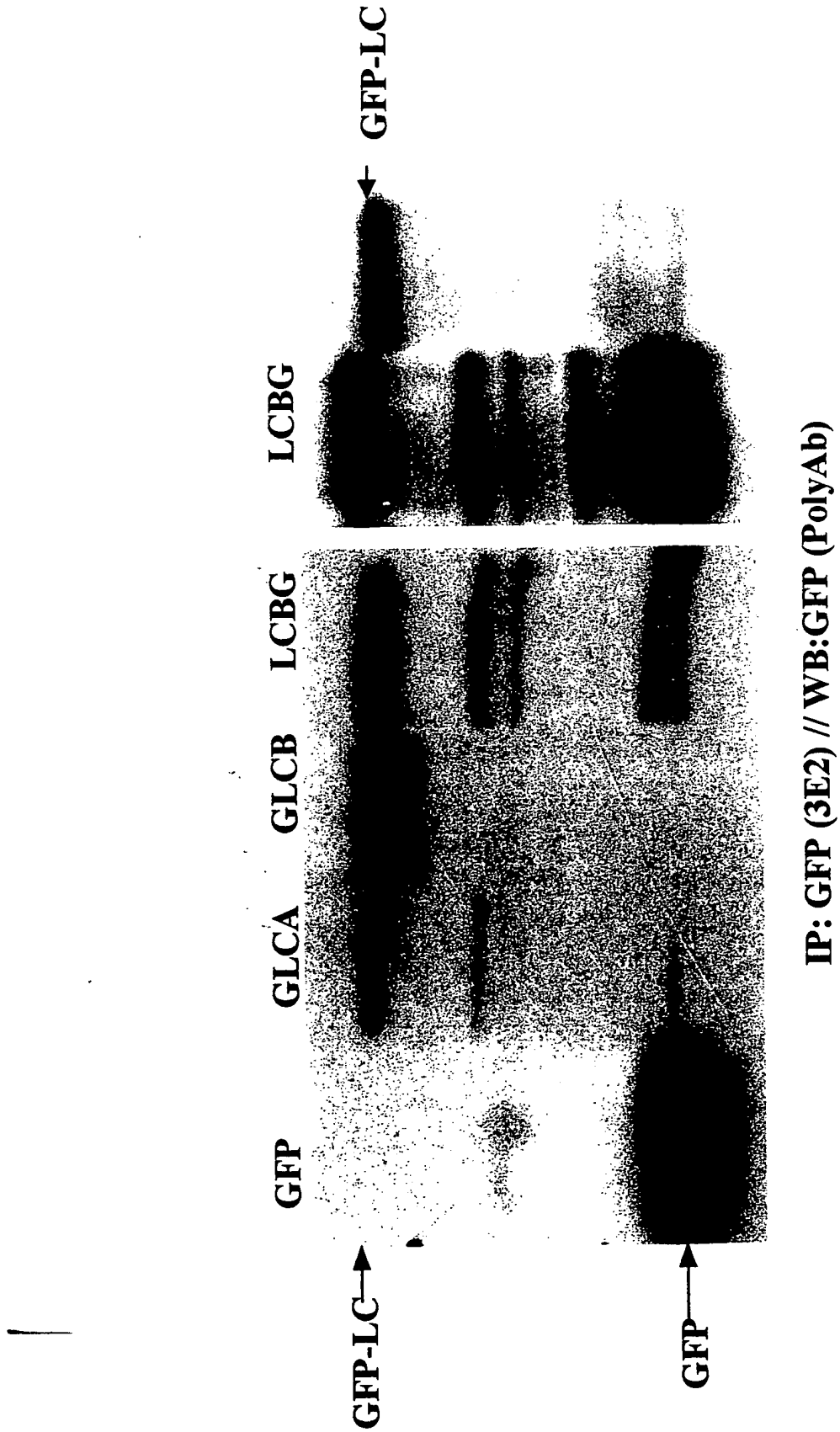

GFP-LC
GFP
GLCA
GLCB
LCBG
LCBG
GFP-LC
GFP
IP: GFP (3E2) // WB:GFP (PolyAb)

FIG. 19

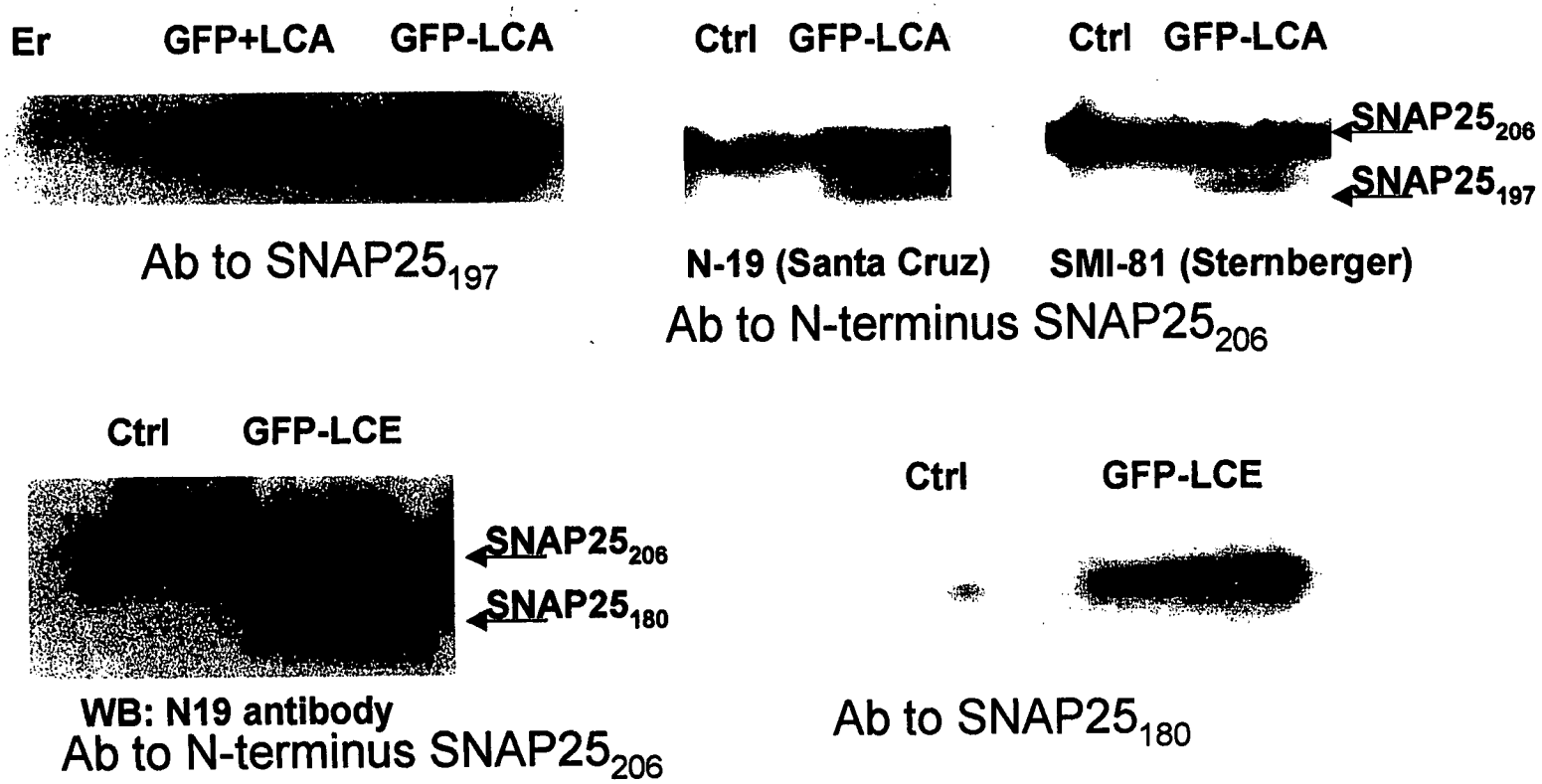

Er
GFP+LCA
GFP-LCA
Ctrl
GFP-LCA
Ctrl
GFP-LCA
SNAP25206
SNAP25197
Ab to SNAP25197
N-19 (Santa Cruz)
SMI-81 (Sternberger)
Ab to N-terminus SNAP25206
Ctrl
GFP-LCE
SNAP25206
SNAP25180
WB: N19 antibody
Ab to N-terminus SNAP25206
Ctrl
GFP-LCE
Ab to SNAP25180

FIG. 20

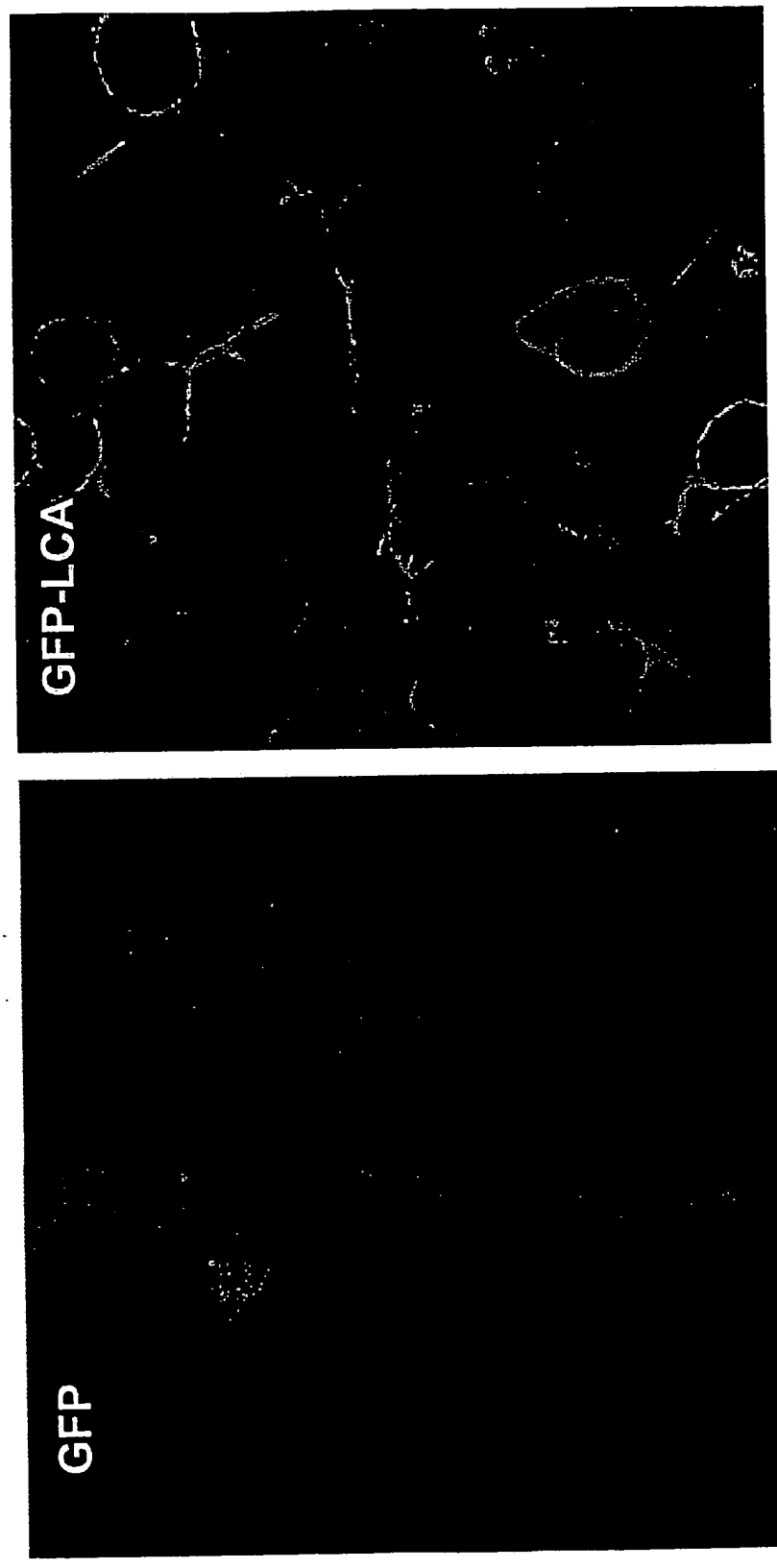


FIG. 21

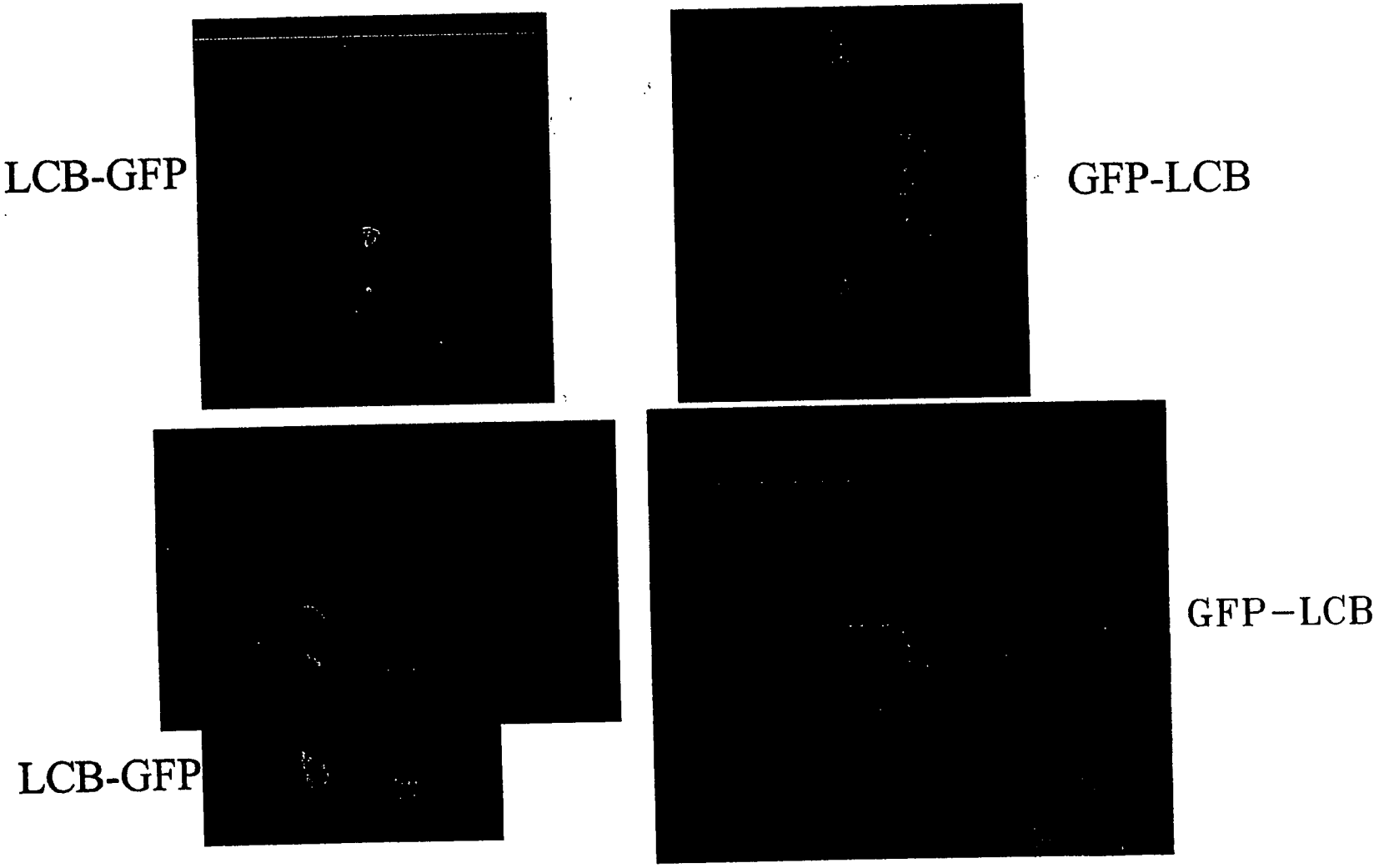


FIG. 22

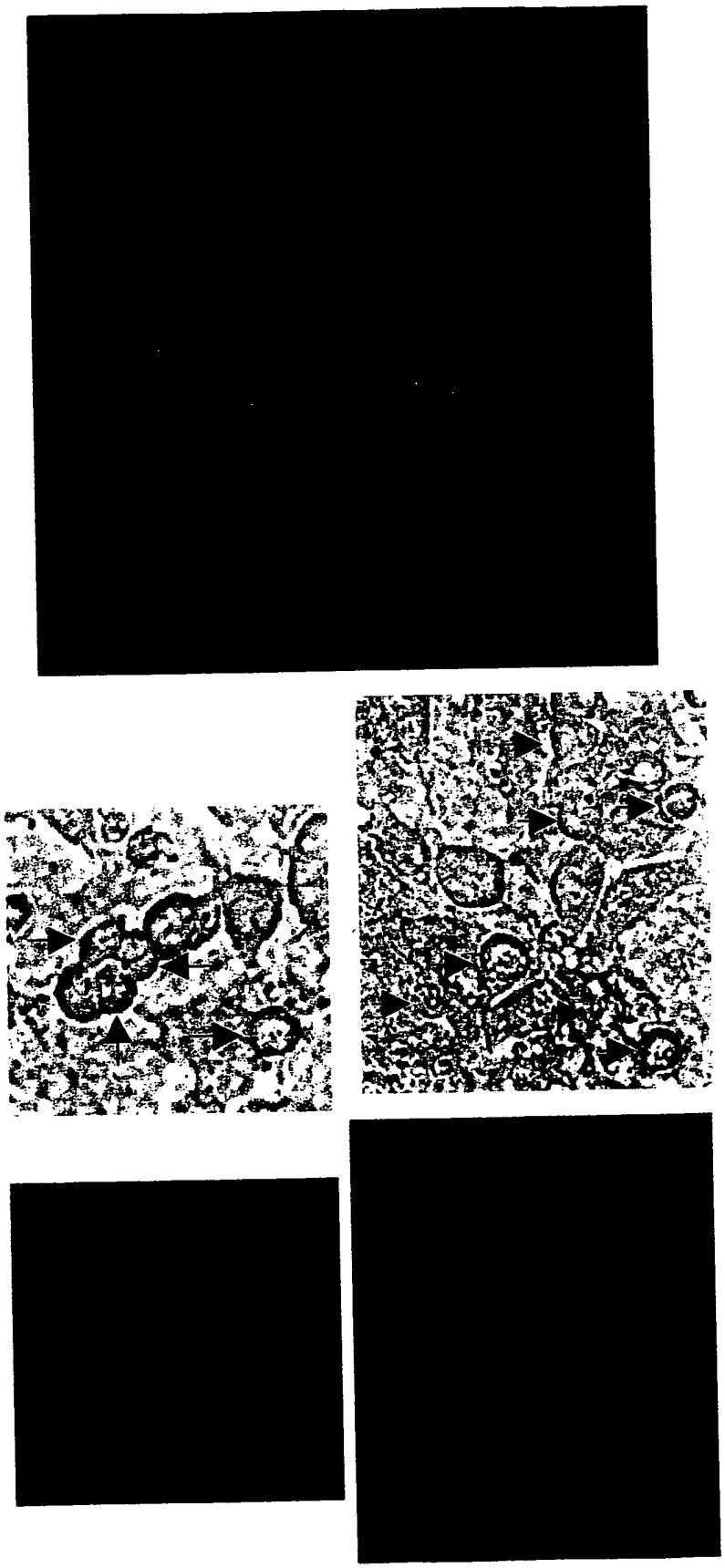


FIG. 23

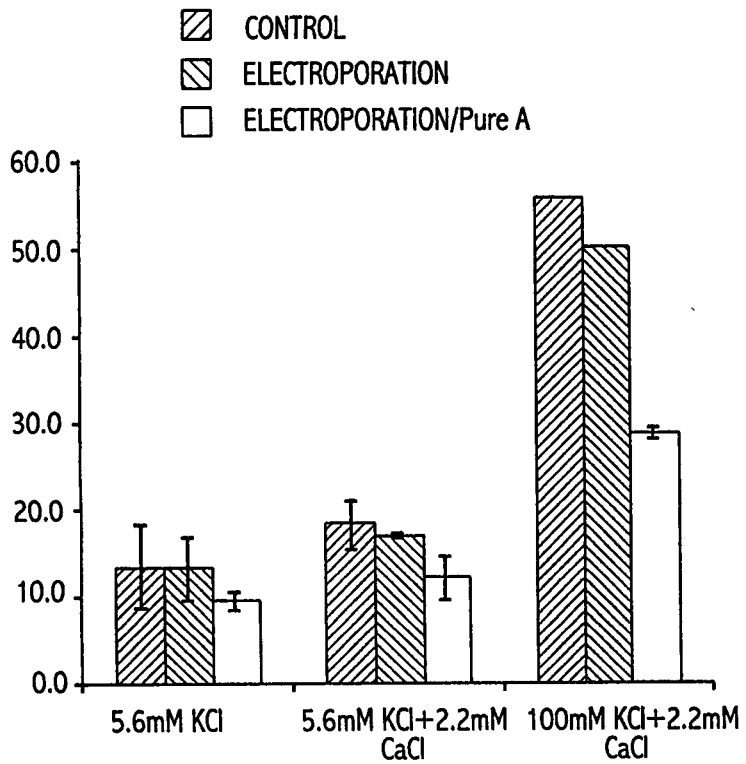


FIG. 24A

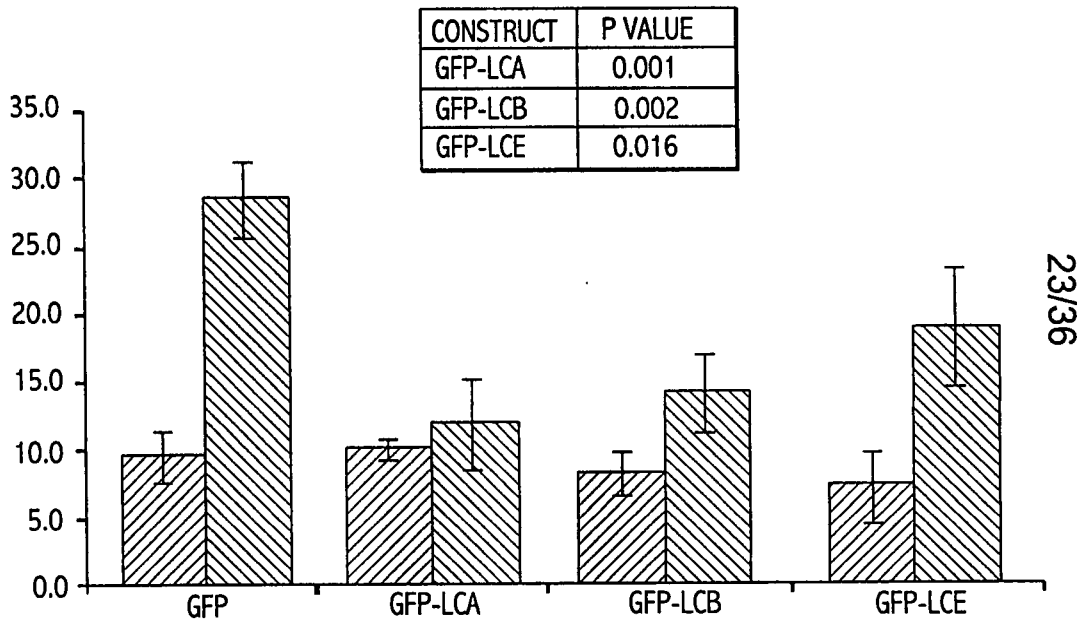
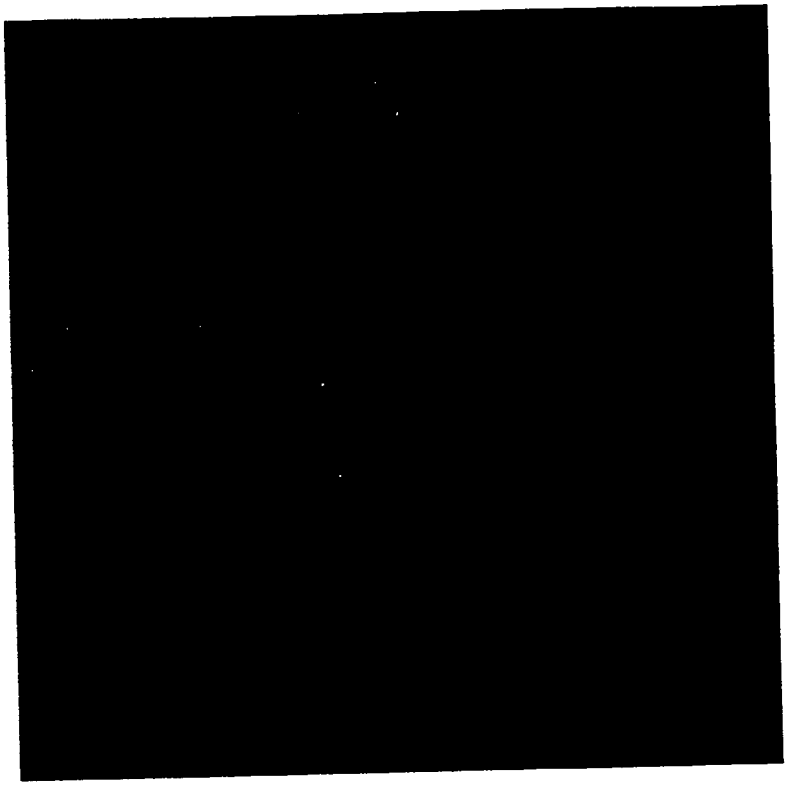
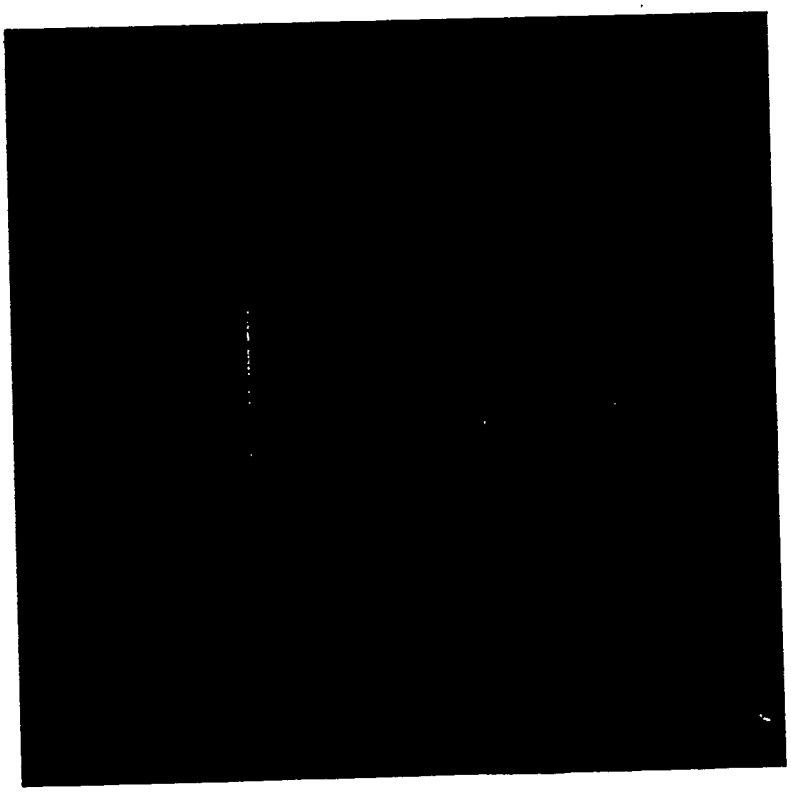


FIG. 24B



HEK293T



HeLA

FIG. 25

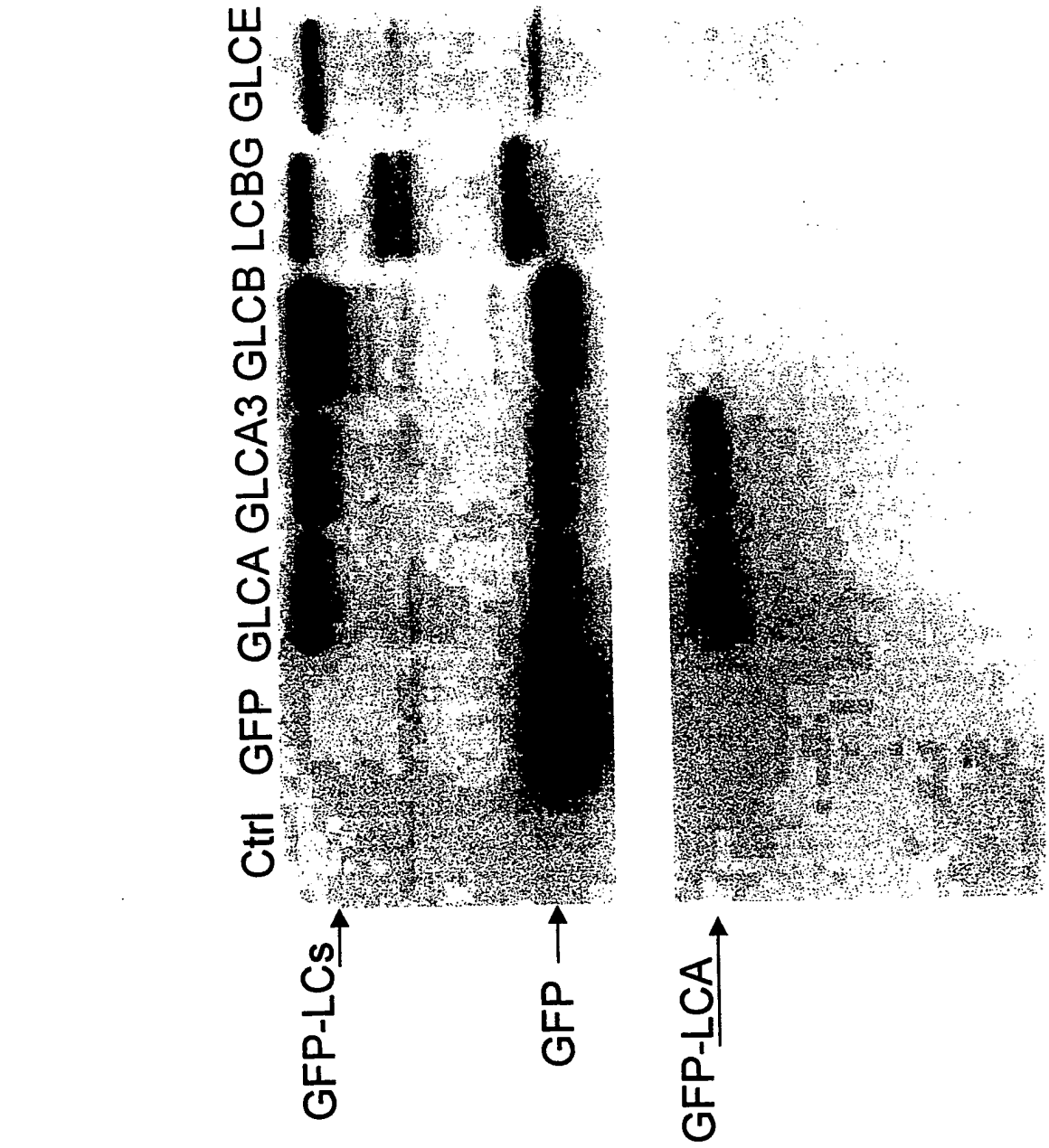


FIG. 26

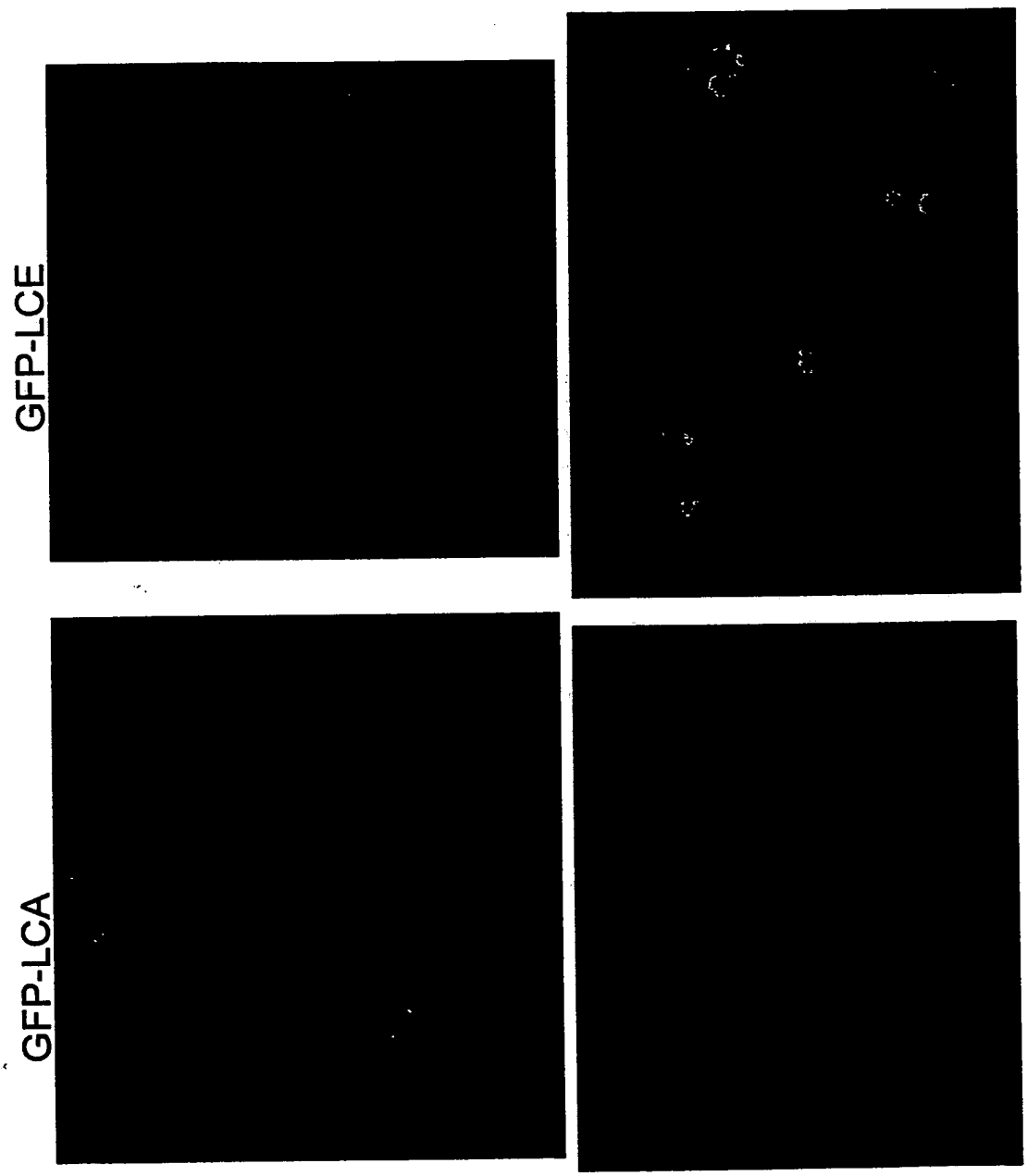


FIG. 27

FIG. 28

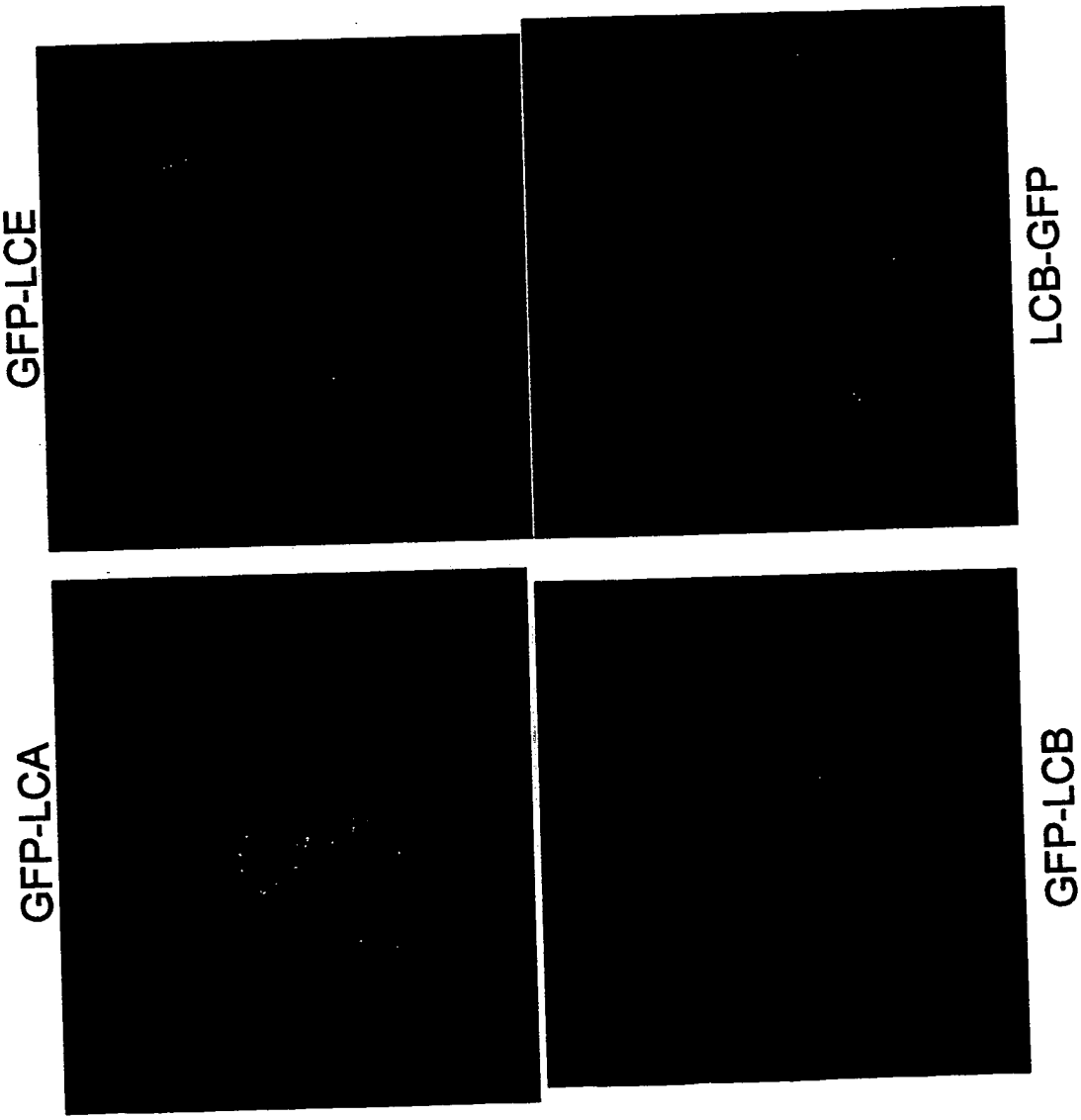


FIG. 29

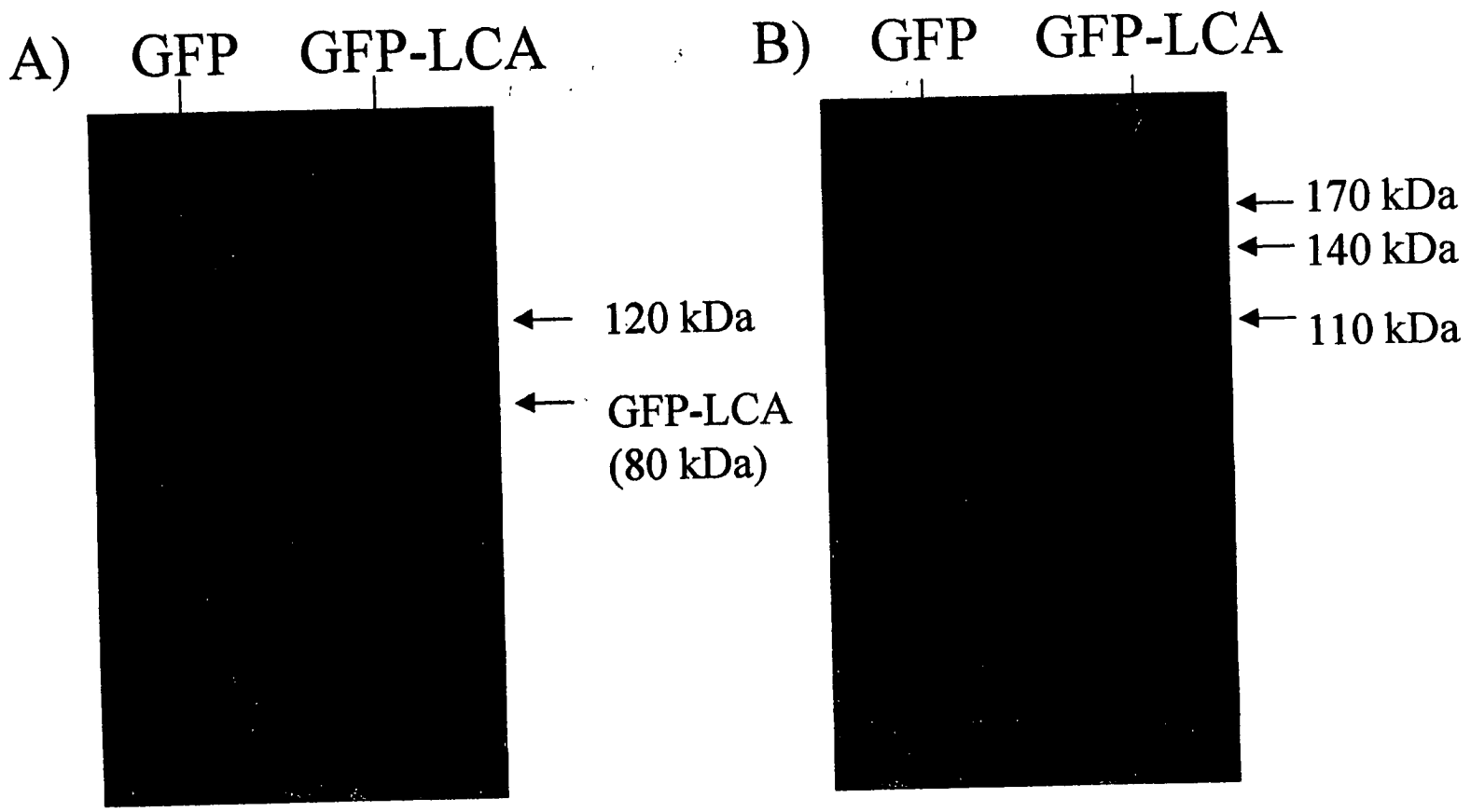

A)
GFP
GFP-LCA
120 kDa
GFP-LCA
(80 kDa)
B)
GFP
GFP-LCA
170 kDa
140 kDa
110 kDa

FIG. 30

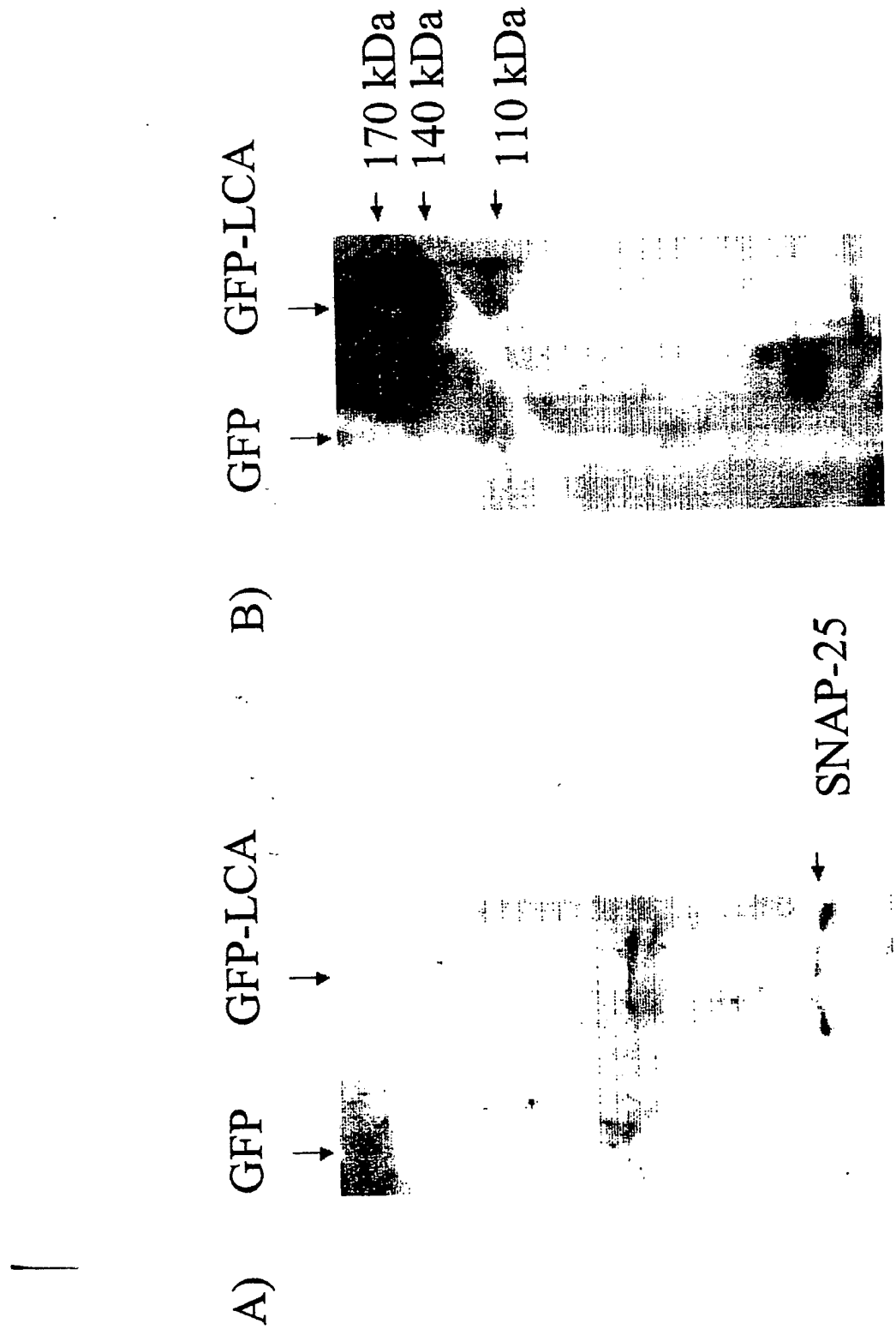


FIG. 31

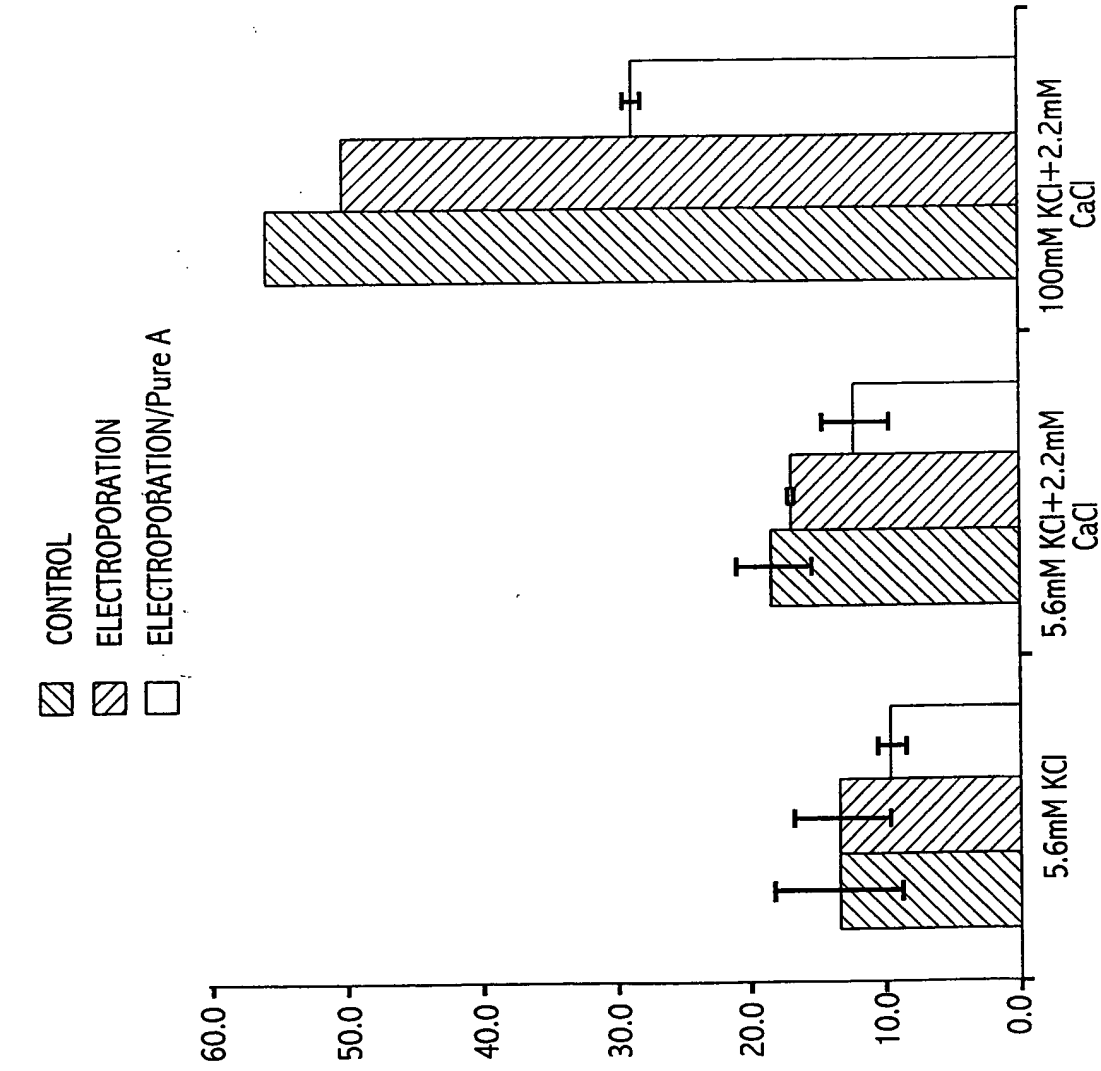

CONTROL
ELECTROPORATION
ELECTROPORATION/Pure A
60.0
50.0
40.0
30.0
20.0
10.0
0.0
5.6mM KCl
5.6mM KCl+2.2mM CaCl
100mM KCl+2.2mM CaCl

FIG. 32

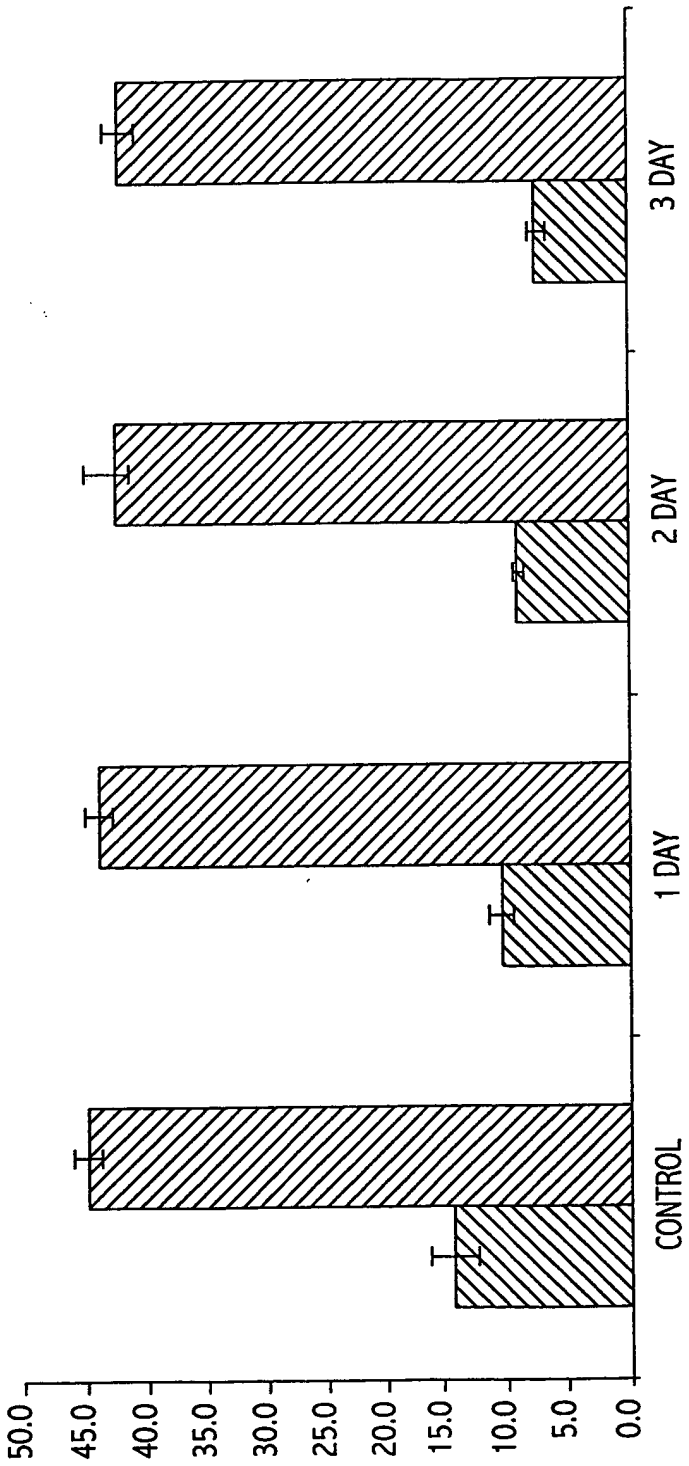

50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
CONTROL
1 DAY
2 DAY
3 DAY

FIG. 33

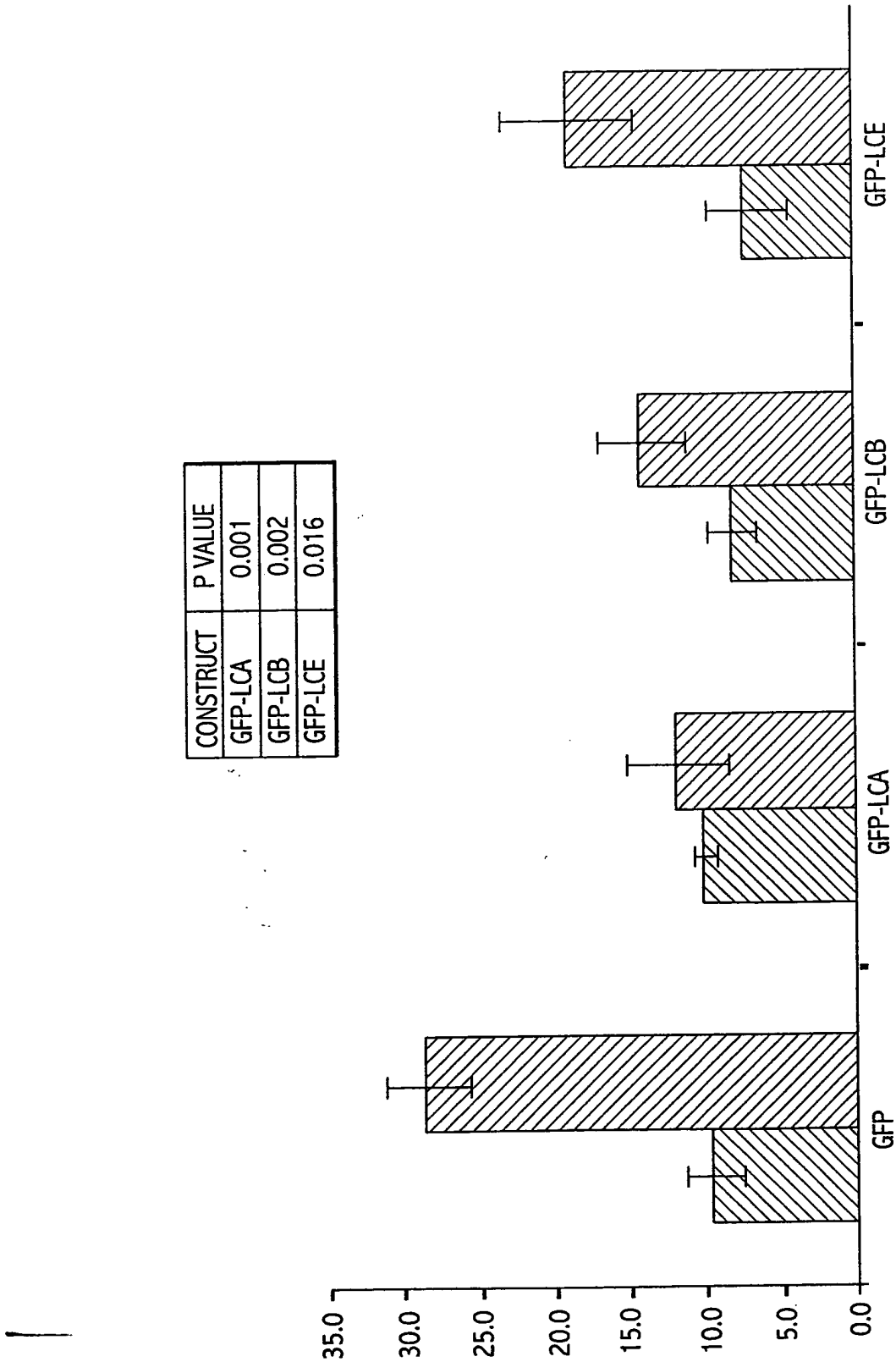

35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
GFP
GFP-LCA
GFP-LCB
GFP-LCE
CONSTRUCT | P VALUE
GFP-LCA | 0.001
GFP-LCB | 0.002
GFP-LCE | 0.016

FIG. 34

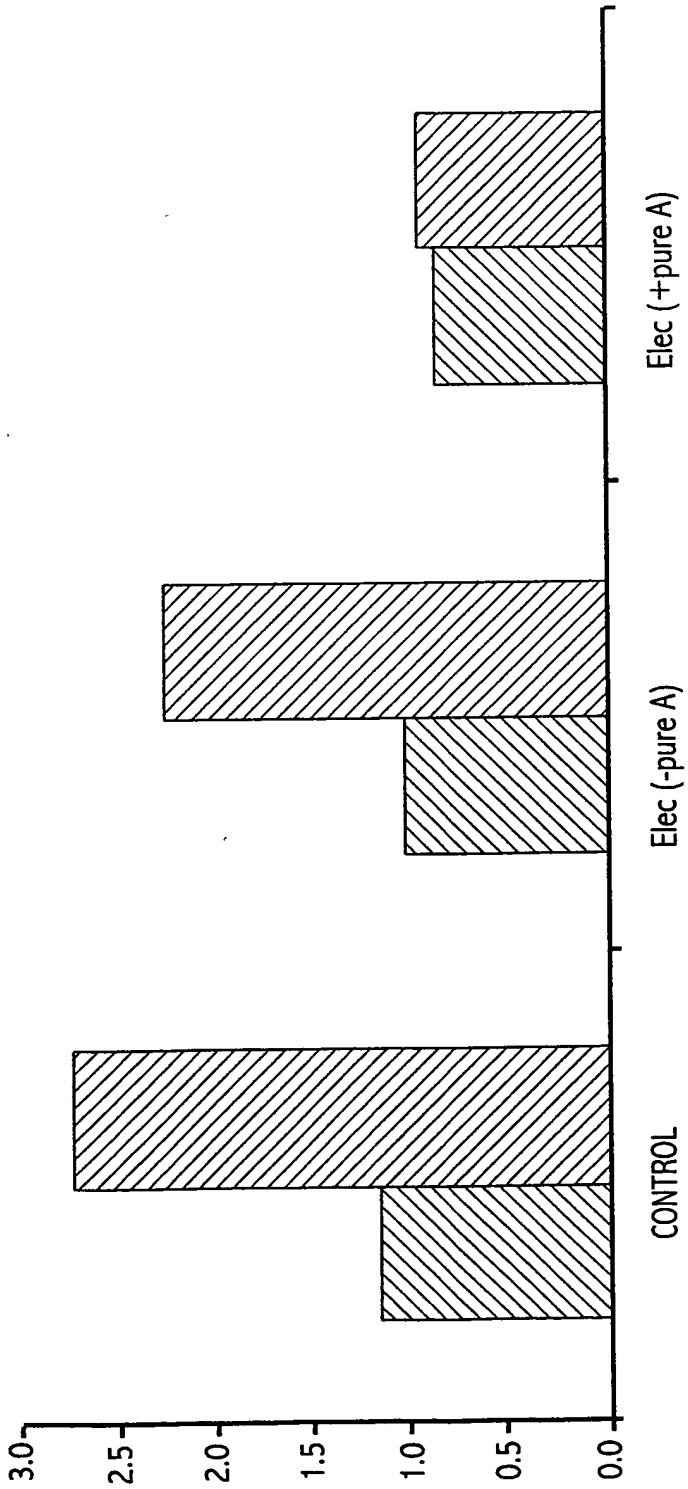

5.6 mM GLUCOSE
25.2 mM GLUCOSE
3.0
2.5
2.0
1.5
1.0
0.5
0.0
CONTROL
Elec (-pure A) TREATMENT
Elec (+pure A)

FIG. 35

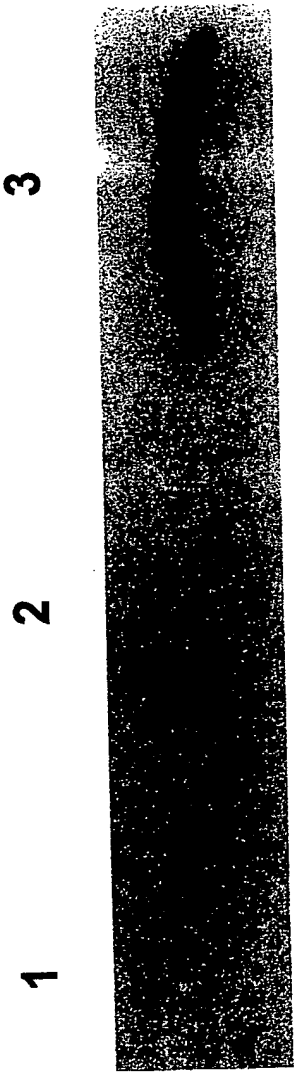


FIG. 36

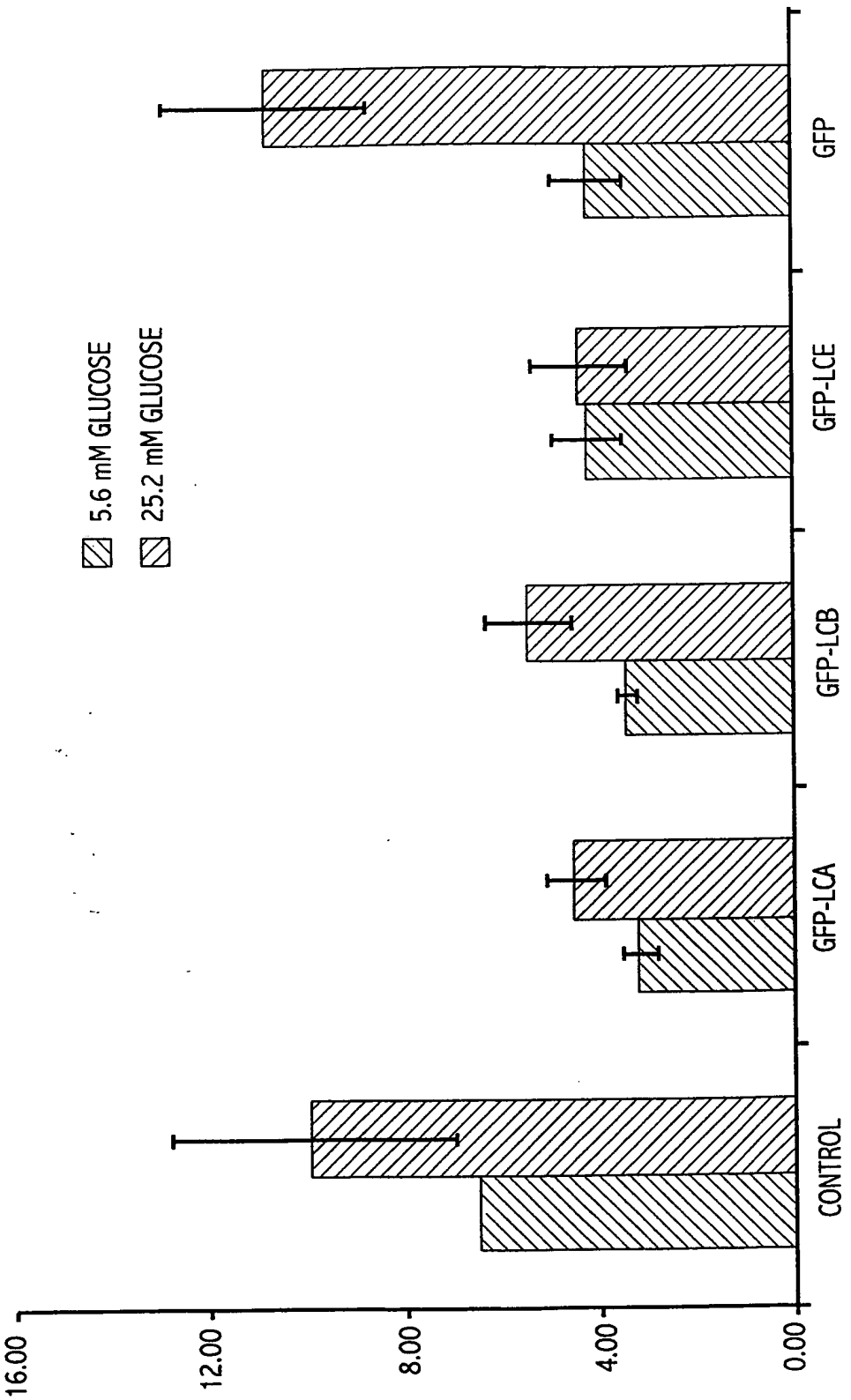

16.00
12.00
8.00
4.00
0.00
5.6 mM GLUCOSE
25.2 mM GLUCOSE
CONTROL
GFP-LCA
GFP-LCB
GFP-LCE
GFP

FIG. 37

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